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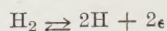
Preparation of Cell-free Solutions of Hydrogenase.

M. BOVARNICK. (Introduced by Augustus B. Wadsworth.)

*From the Division of Laboratories and Research, New York State Department of Health, Albany.*

In certain bacterial species, including *B. coli*, *Clostridium sporogenes*, and others, enzyme systems have been described which catalyze the reduction by molecular hydrogen of various substrates such as methylene blue, nitrate, fumarate, oxygen, and some amino

acid.<sup>1,2</sup> It has been shown<sup>3,4</sup> that the enzyme of *B. coli* which activates molecular hydrogen, hydrogenase, catalyzes in a completely reversible manner the reaction



The enzyme preparations used in previous studies have been suspensions of intact growing or resting bacterial cells; extraction of the enzymes involved resulted in loss of activity.<sup>5</sup>

As a basis for further study of this enzyme the observations reported in this paper seemed of interest, namely, that the system catalyzing the reduction of methylene blue by hydrogen is quite stable, and that active dry preparations and cell-free solutions can readily be secured, as evidenced by data in the accompanying table.

An 18-hour broth culture of *B. coli communior*, No. 142, was washed twice with saline by centrifugation and resuspended in 0.1 M phosphate buffer, pH 7.0. The final volume was one-thirtieth that of the original culture. Active dry powders were obtained by pouring this suspension into 20 volumes of cold acetone and rapidly filtering by suction. Cell-free solutions were obtained by filtering a 16-day autolysate through Mandler filters. Other methods of lysing the cells, such as alternate freezing and thawing, and prolonged exposure to high salt concentration followed by rapid dilution, have yielded solutions that were active but difficult to filter.

The reactions were carried out at room temperature and at pH 7.0

TABLE I.  
Catalysis of Reduction of Methylene Blue by Hydrogen.

Preparation	Gas	Methylene blue (1 ml)	Time required for decoloration
Washed <i>B. coli</i> suspended in 4 ml phosphate buffer	H	1/5000	8 min
Same	N	"	>90 "
Washed <i>B. coli</i> susp. heated 100°C 20 min	H	"	> 5 hr
Acetone-dried preparation suspend- ed in 4 ml phosphate buffer	H	1/1000	20-25 min
Same	N	"	> 6 hr
Suspension of acetone-dried prep- aration heated 100°C 20 min	H	"	> 6 "
Mandler filtrate	H	1/5000	25 min
" "	N	"	>12 hr
" " heated 100°C 20 min	H	"	>12 hr

<sup>1</sup> Stephenson, Marjory, and Stickland, L. H., *Biochem. J.*, 1931, **25**, 205.

<sup>2</sup> Hoogerheide, J. C., and Kocholaty, Walter, *Biochem. J.*, 1938, **32**, 949.

<sup>3</sup> Green, D. E., and Stickland, L. H., *Biochem. J.*, 1934, **28**, 898.

<sup>4</sup> Farkas, A., Farkas, L., and Yudkin, J., *Proc. Roy. Soc. London, Series B*, 1934, **115**, 373.

<sup>5</sup> Stickland, L. H., *Biochem. J.*, 1929, **23**, 1187.

(0.1 M phosphate buffer), either in modified Thunberg tubes or, more conveniently, by bubbling the gases through the solutions in test tubes provided with two-holed rubber stoppers bearing inlet and outlet tubes. Amounts of the enzyme preparation were used that corresponded to 4 ml of the original phosphate suspension. The gases used were deoxygenated by passing over hot reduced copper filings.

In the presence of active preparations the dye was reduced under an atmosphere of hydrogen but not under nitrogen. Heating for twenty minutes at 100°C destroyed the activity of the enzyme.

*Summary.* Stable dry powders and cell-free solutions that possess hydrogenase activity have been prepared from cultures of *B. coli communior*.

## 13082

## Effect of Gramicidin on Metabolism of Bovine Spermatozoa.\*

GERTRUDE HENLE AND CHARLES A. ZITTLE. (Introduced by Stuart Mudd.)

*From the Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia.*

Gramicidin,<sup>†,‡</sup> a bactericidal agent for gram-positive organisms,<sup>1</sup> seems to have a wider range of activity than the name indicates. It acts as a hemolytic agent in low concentrations,<sup>2,§</sup> and it is highly

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\* This work has been aided by a grant from the National Committee on Maternal Health, Inc.

† The preparation of crude and purified gramicidin, and purified tyrocidine, used in these studies were obtained through the kindness of Dr. R. J. Dubos, who independently suggested this investigation. We are indebted also to Dr. Dubos for reading this paper before publication.

‡ Crude gramicidin has recently been renamed tyrothricin by Hotchkiss and Dubos<sup>4</sup> and the name gramicidin retained for one of the purified components. The other principal component has been purified also and named tyrocidine; it exhibits bactericidal activity against both Gram positive and Gram negative organisms.

<sup>1</sup> Dubos, R. J., *J. Exp. Med.*, 1939, **70**, 1, 11; Dubos, R. J., and Cattaneo, C., *ibid.*, 249; Dubos, R. J., *Ann. Int. Med.*, 1940, **13**, 2025.

<sup>2</sup> Heilman, D., and Herrell, W. E., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 182.

§ Hotchkiss and Dubos<sup>5</sup> recently have stated that tyrocidine is hemolytic but that purified gramicidin is not hemolytic. Further studies<sup>6</sup> have confirmed the hemolytic effect of gramicidin in saline or buffer solutions<sup>2</sup> but have shown that hemolysis is completely inhibited by small amounts of glucose.



toxic if injected into animals by the intravenous route.<sup>3</sup> The effect of gramicidin on mammalian cells other than erythrocytes has not been investigated to our knowledge.

Bovine spermatozoa, which are being studied extensively in this laboratory, appeared to be suitable material for such an investigation, since they can readily be obtained in pure suspension, and, being a type of mammalian cell, might be expected to throw some light on the observed toxicity of this substance in animals. In the following we shall describe the action of gramicidin on the metabolism and the motility of bovine spermatozoa. Some data are available for the normal respiration and glycolysis of these cells;<sup>7-10</sup> further information is being collected in this laboratory at present.

*Experimental.* Spermatozoal suspensions were prepared from the bovine epididymis as described elsewhere.<sup>11</sup> Ringer solution containing a final concentration of 0.2% glucose with or without the addition of M/15 phosphate buffers (added in ratio of 1 to 4) of pH 5.3 to 8.0 served as medium for the determination of the oxygen consumption, while Ringer glucose with sodium bicarbonate (0.025 M) was used for the measurement of the glycolysis. Alcoholic solutions of the gramicidin preparations were added to Ringer solution and appropriate volumes of the resulting suspension used.

Oxygen consumption and anaerobic glycolysis were measured by the direct method of Warburg<sup>12</sup> under air and under nitrogen with 5% CO<sub>2</sub>, respectively. In a number of experiments the respiratory quotient (RQ) and at the same time the aerobic glycolysis were determined in Dixon-Keilin flasks<sup>12</sup> attached to Warburg manometers filled with Clerici solution. The results were calculated according to the second method of Dickens and Simer.<sup>12</sup> In these experiments Ringer bicarbonate solution was used as medium, the suspensions being in equilibrium with a mixture of oxygen and 5% CO<sub>2</sub>. Two ml of suspensions containing 150 to 700 million spermatozoa per ml were used per vessel. All of the experiments were performed at 37°C.

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<sup>3</sup> McLeod, C. A., Mirick, G. S., and Curnen, E. C., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 461.

<sup>4</sup> Hotchkiss, R. D., and Dubos, R. J., *J. Biol. Chem.*, 1940, **136**, 803.

<sup>5</sup> Hotchkiss, R. D., and Dubos, R. J., *J. Biol. Chem.*, Proceedings, 1941, lxiii.

<sup>6</sup> Dubos, R. J., and Hotchkiss, R. D., *J. Exp. Med.*, 1941, **73**, 629.

<sup>7</sup> Redenz, E., *Biochem. Z.*, 1932-33, **257**, 234.

<sup>8</sup> Iwanow, E. E., *Biochem. Z.*, 1935, **278**, 101.

<sup>9</sup> Comstock, R. E., *J. Exp. Zool.*, 1940, **81**, 147.

<sup>10</sup> Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, 1941, **138**, 195.

<sup>11</sup> Henle, W., *J. Immunol.*, 1938, **34**, 325.

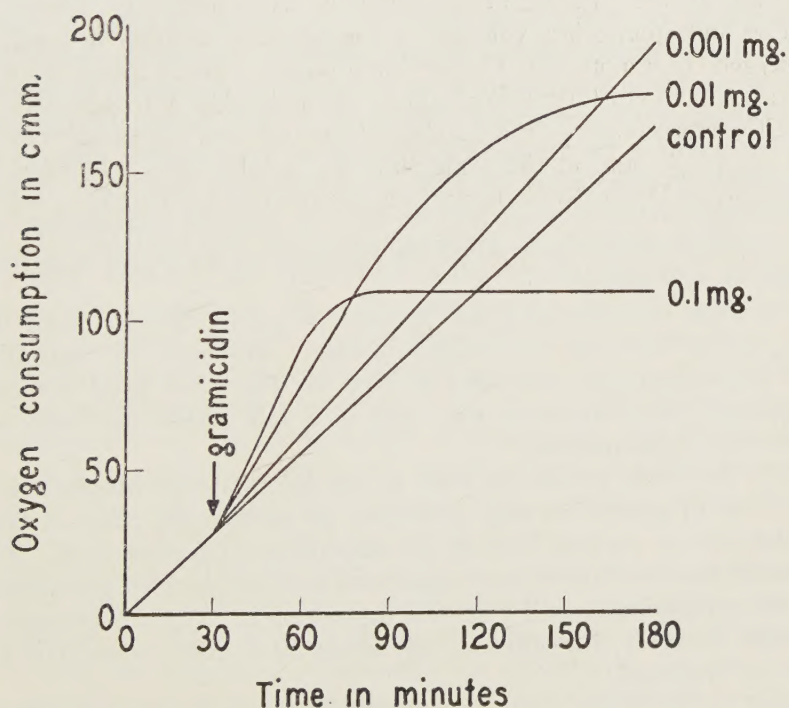
<sup>12</sup> Dixon, M., *Manometric Methods*, Cambridge, 1934.



It was found that small amounts of the crude preparation of gramicidin altered strikingly the oxygen consumption of bovine spermatozoa. Fig. 1 shows the effect of various concentrations of this agent on 400 million spermatozoa suspended in Ringer glucose (pH 6.0) over a period of 150 minutes. Concentrations of 0.1 mg and 0.01 mg of crude gramicidin per ml of suspension caused an initial stimulation of the oxygen uptake, but the respiration fell off rather abruptly after 30 and 90 minutes, respectively. The smallest amount of gramicidin used (0.001 mg per ml suspension) caused only a moderate stimulation of the oxygen consumption, but there was no decrease during the period of observation. As the suspensions of gramicidin were prepared from alcoholic solutions, control experiments were made with comparable amounts of alcohol. No influence on the oxygen uptake or on the motility of the spermatozoa was noticed.

In cases where the oxygen uptake had ceased, the spermatozoa were immobile whereas the cells in the control suspensions were

Fig.1



Influence of gramicidin on the oxygen consumption of bull spermatozoa suspended in Ringer glucose.

quite active. In the experiments where a stimulation of respiration was obtained by the addition of a small amount of gramicidin, the spermatozoa had about the same activity as the control suspensions.

This action of gramicidin on the respiration of spermatozoa suspended in Ringer glucose which had a pH of about 6.0 was also observed when the spermatozoa were suspended in a medium containing phosphate buffers of pH 5.3 and 6.6. When alkaline phosphate buffers (pH 7.9) were added to the Ringer solution, a medium in which the spermatozoa showed maximal respiration,<sup>13</sup> the addition of gramicidin caused a stimulation of oxygen consumption only, and there was no decrease in the uptake during the 4 hours of observation. In a few cases a decrease occurred but only when enough acid had been produced by the cells to lower the pH considerably.

For the simultaneous determination of the  $O_2$  consumed and the  $CO_2$  produced by the technic mentioned the spermatozoa were suspended in Ringer bicarbonate solution. Although the pH of this medium was approximately 7.5 the oxygen consumption of the spermatozoa in this case was reduced 70 to 80% by the addition of gramicidin (Table I), when measured at the end of one hour. Therefore, the stimulating action of gramicidin in alkaline phosphate buffer unaccompanied by final inhibition may not be due to the pH alone, for in bicarbonate at the same pH the depression in oxygen consumption is considerable. However, a direct comparison cannot be made, for in the former experiments air was present, whereas in the latter an atmosphere of 95%  $O_2$  and 5%  $CO_2$  was used. These differing conditions must also be kept in mind in considering the glycolysis experiments in which the inhibition of glycolysis by gramicidin is only partial.

In these experiments it was found that gramicidin decreased the  $RQ$  ( $CO_2/O_2$ ) from 1.0 to 0.8 in 2 cases. However, the reduction of respiration was such that in view of the small total volumes measured the differences may very well fall within the limits of accuracy of the method.<sup>12</sup>

As the crude gramicidin used in the original experiments was a mixture of gramicidin and tyrocidine, we studied the effect of both substances in purified form on the respiration of spermatozoa. The results described above were duplicated with the purified gramicidin, while tyrocidine showed a definite depression in oxygen consumption which, however, amounted to only about 10 to 20%, when 0.05 mg per ml was used.

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<sup>13</sup> Henle, G., and Zittle, C. A., unpublished data.



TABLE I.

Influence of Gramicidin on Respiratory Quotient and Aerobic Glycolysis of Bull Spermatozoa.

Exp.	Millions of sperm per ml	Concen. gramicidin mg/ml suspension	RQ		$Z_{O_2}$			$Z_M^{O_2}$		
			Control	Gram-icidin	Control	Gram-icidin	% de-pression	Control	Gram-icidin	% de-pression
1	650	0.09 crude	1.02	0.80	27.1	6.5	76	36.6	22.8	38
2	335	0.10 "	1.01	0.85	27.9	7.0	75	30.0	25.5	15
3	200	0.05 "	0.98	1.10	16.2	5.1	69	42.7	23.4	45
4	180	0.02 "						48.0	31.0	35
5	155	0.05 purif.						41.6	17.4	58

$$Z_{O_2} = \frac{\text{mm}^3 O_2}{100 \times 10^6 \text{ sperm/hour}}$$

$$Z_M^{O_2} = \frac{\text{mm}^3 CO_2 \text{ (lactic acid)}}{100 \times 10^6 \text{ sperm/hour}}$$

Some of the experiments for the determination of glycolysis under aerobic conditions are cited in Table I. The values obtained with gramicidin showed a reduction in comparison with the controls, but the glycolysis continued in most cases over the period of observation. The inhibition of glycolysis caused by 0.05 mg of purified gramicidin per ml was on the average 42.5% (6 experiments). Purified tyrocidine in the same concentration caused only a depression of about 15% of the control values.

Similar experiments were conducted under anaerobic conditions. The inhibition of glycolysis amounted to about 45%, when the spermatozoa had been in contact with gramicidin for one hour, and in 2 to 3 hours the glycolysis was depressed up to 75% of the controls.

The motility of the spermatozoa in bicarbonate medium was greatly impaired by gramicidin under aerobic as well as under anaerobic conditions. The cells were found to be immobile in some cases, in others they no longer showed forward movements. The spermatozoa of the control suspensions were very actively motile in some experiments; in others, where the pH had dropped in a period of several hours the spermatozoa were less motile, but their activity could be restored by addition of a small amount of sodium bicarbonate to the suspension. Spermatozoa, whose motility had been impaired by gramicidin, could not be reactivated.

*Discussion.* The stimulation of the respiration of spermatozoa in moderately acid solution by small amounts of gramicidin, and the toxic action of larger amounts has been paralleled with other kinds of cells and reagents. The action of substituted phenols on the respiration of the fertilized eggs of *Arbacia punctulata*<sup>14</sup> is similar.

<sup>14</sup> Krah1, M. E., and Clowes, G. H. A., *J. Gen. Physiol.*, 1940, **23**, 413.

and the action of detergents on the respiration of bacteria has in some cases shown this phenomenon also.<sup>15</sup> In the latter studies also a considerable effect of pH was observed; the cationic detergents were most active in the alkaline range, *i. e.*, when least ionized, and the anionic in the acid range. The degree of ionization can not explain the observed effect of pH on the activity of gramicidin, for gramicidin has recently<sup>4</sup> been described as a neutral polypeptide containing neither free amino nor carboxyl groups.

The inability of the purified gramicidin to hemolyze red blood cells in the presence of small amounts of glucose<sup>6</sup> eliminates this as an explanation of its toxic action in animals. The effect on spermatozoa we have observed suggests that perhaps its harmful action is exerted through the enzymes involved in respiration and glycolysis. Further studies will be necessary to determine the particular enzymes affected.

The action of gramicidin on spermatozoa seems to be of about the same magnitude as its action on pneumococci. Dubos has found 0.01 mg completely inhibits the growth of  $10^9$  pneumococci within 2 hours; an enormous stimulation of the oxygen consumption of Gram-positive organisms occurs.<sup>16</sup> No pH effect was observed.

*Summary.* Crude and purified gramicidin after initial stimulation inhibits the oxygen consumption of bovine spermatozoa completely in Ringer phosphate of acid pH and renders the cells immobile. By the use of sufficiently small amounts only stimulation is observed during the period of the experiment. In alkaline phosphate buffer only the increase of oxygen consumption is noted. However, in Ringer bicarbonate medium of the same pH, the respiration again is greatly inhibited; aerobic as well as anaerobic glycolysis are depressed on the average 40% and the motility of the spermatozoa is markedly impaired. Tyrocidine, in the concentrations studied, caused a small reduction in the oxygen uptake and the glycolysis.

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<sup>15</sup> Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **73**, 249.

<sup>16</sup> Dubos, R. J., personal communication.



## 13083 P

# Activity of Alpha Tocopherol in Preventing Antagonism Between Linoleic and Linolenic Esters and Carotene.

W. C. SHERMAN. (Introduced by C. A. Elvehjem.)

*From the Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn, Alabama.*

The author has shown<sup>1, 2</sup> that the methyl esters of linoleic and linolenic acids interfere with the utilization of carotene and vitamin A by vitamin-A deficient rats. It was found that this antagonism can be counteracted by the addition of soybean oil and is not apparent if sufficiently large amounts of carotene are fed.

Subsequent investigations have revealed that the antagonism between the unsaturated fatty acid esters and carotene can be prevented by the unsaponifiable fraction of soybean oil or by  $\alpha$ -tocopherol; but not by choline, ethanolamine, or soybean lecithin or cephalin.

The growth obtained with carotene and  $\alpha$ -tocopherol was only slightly better than that with carotene alone, but the addition of  $\alpha$ -tocopherol to carotene and methyl linolate or linolenate gave a pronounced growth response. The antagonism between methyl linolenate and carotene was more pronounced than that between methyl linolate and carotene. The addition of  $\alpha$ -tocopherol gave better growth with methyl linolate than with methyl linolenate.

It is improbable that  $\alpha$ -tocopherol acts as a simple chemical anti-

TABLE I.  
Growth Responses of Vitamin A-Deficient Rats.\*

Daily supplements	No. of rats	Avg gain end of 7th week, g
2 $\gamma$ Carotene	8	31
2 $\gamma$ " and 1 mg $\alpha$ -tocopherol	4	46
2 $\gamma$ " + .05 g methyl linolate	10	19
2 $\gamma$ " + .05 g methyl linolate + unsaponifiable matter from 0.1 ml soybean oil	9	81
2 $\gamma$ " + .05 g methyl linolate + 1 mg $\alpha$ -tocopherol	20	80
2 $\gamma$ " + .05 g methyl linolenate	4	9
2 $\gamma$ " + .05 g methyl linolenate + 1 mg $\alpha$ -tocopherol	8	48

\*All rats received a diet of the following composition during the depletion and experimental periods: casein (alcohol extracted) 18, salt mixture No. 186 (*J. Biol. Chem.*, 1930, **89**, 199) 4, Northwestern yeast, irradiated 6, and sucrose 72.

1 Sherman, W. C., *J. Biol. Chem., Proc.*, 1940, **133**, 89.

2 Sherman, W. C., *J. Nutrition*, in press.

oxidant since *in vitro* experiments<sup>2</sup> have shown that the unsaturated fatty acid esters cause no direct destruction of carotene with the method of feeding employed. It appears that in the absence of  $\alpha$ -tocopherol there is a physiological antagonism between unsaturated fatty acids and carotene which results in the inefficient utilization of carotene.

### 13084 P

#### Immunological Properties of an Antibody Containing a Fluorescent Group.\*

ALBERT H. COONS,<sup>†</sup> HUGH J. CREECH AND R. NORMAN JONES.  
(Introduced by J. F. Enders.)

*From the Department of Bacteriology and Immunology, Harvard Medical School, and the Chemical Laboratory, Harvard University.*

Previous investigations involving chemical derivatives of antibodies usually have been planned either to establish the protein nature of the antibody molecule, or to elucidate the influence of specific polar groups on the mechanism of the antigen-antibody reaction. Reiner,<sup>1</sup> however, prepared serologically active atoxyl-azo conjugates of antipneumococcus I and II antibodies, and suggested that they might be useful in quantitative studies of antigen-antibody reactions. Marrack<sup>2</sup> allowed anti-typhoid and anti-cholera sera to react with diazotized benzhidine-azo-R-salt, and demonstrated that homologous organisms were specifically colored pink by the chemically modified antibodies.

The objective of the present investigation is the development of a method by which antigenic substances could be revealed in mammalian tissues.

One of us (A.H.C.)<sup>‡</sup> has repeated Marrack's experiment with antipneumococcus II and III rabbit sera,<sup>§</sup> and has found that sus-

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\* Aided in part by a grant from the International Cancer Research Foundation.

† Fellow in the Medical Sciences of the National Research Council.

<sup>1</sup> Reiner, L., *Science*, 1930, **72**, 483.

<sup>2</sup> Marrack, J., *Nature*, 1934, **133**, 292.

‡ Unpublished data.

§ Lederle's concentrated therapeutic rabbit sera were used in this work. The pneumococcus-antipneumococcus system was chosen for convenience in developing the method.



pensions of these organisms were agglutinated and colored by the specific azo-serum. The color obtained, however, was insufficiently intense to render the method suitable for the purpose in mind.

Conjugates prepared by the interaction of isocyanates of polynuclear aromatic hydrocarbons with several proteins have been shown to be highly fluorescent.<sup>3</sup> Since such fluorescent labels might be easier to distinguish than colored groups, a  $\beta$ -anthryl-carbamido derivative of antipneumococcus III rabbit serum was prepared. Conjugation was obtained in an aqueous-dioxane medium by the interaction of  $\beta$ -anthryl isocyanate with the serum. Experimental conditions were chosen such that a minimum alteration of the protein molecule would occur. The anthracene content of the conjugate, determined by ultraviolet spectrophotometry, was two groups per molecule of protein (taking 160,000 as the molecular weight of the protein). This conjugate gives an optically clear solution in physiological saline in a concentration corresponding to 1/10th that of the original serum. It has a faint blue fluorescence in daylight, and an intense blue fluorescence in ultraviolet light, even in very dilute solution. Specifically precipitated by pneumococcus III carbohydrate, it agglutinates Type III organisms in the same titer as the original serum (1/800), fixes complement, and passively sensitizes the guinea pig to anaphylactic shock. Parallel opsonocytophagic tests with the anthracene derivative and the original serum showed equal quantitative sensitization of the organisms. These tests, however, do not prove that the antibody molecules themselves are conjugated with the isocyanate, although the quantitative determinations suggest this.

Accordingly the conjugate (diluted 1/50 in terms of the original serum) was mixed in equal proportions with a similar dilution of unaltered Type II antipneumococcus rabbit serum of approximately equal agglutinating titer. Type II pneumococci were added to one aliquot of this mixture, and Type III pneumococci to a second. Agglutination occurred in both tubes. When these two suspensions were illuminated with ultraviolet light in the fluorescence microscope, the clumps of Type III organisms exhibited a bright blue fluorescence. No fluorescence was seen in the Type II clumps. After centrifugation the organisms were washed with 0.9% saline and re-centrifuged. Again the Type III organisms showed a bright blue fluorescence macroscopically, whereas the Type II organisms did not. Moreover, when Types II and III organisms were dried on different

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<sup>3</sup> Creech, H. J., and Jones, R. N., *J. Am. Chem. Soc.*, 1940, **62**, 1970; *J. Am. Chem. Soc.*, 1941, **63**, 1661, 1670.

parts of the same slide, exposed to the conjugate for 30 minutes, washed in saline and distilled water, and mounted in glycerol, individual Type III organisms could be seen with the fluorescence microscope, whereas the Type II organisms were invisible, although their presence was readily demonstrated at the same focus with visible light. Non-specific adsorption and mechanical occlusion of fluorescent molecules during agglutination would thus seem to be eliminated. Although the isocyanate undoubtedly reacts with other protein molecules in the antibody solution, it seems clear that the antibody molecules also have undergone conjugation without demonstrable impairment of specific function.

Mammalian connective tissue normally exhibits a blue fluorescence which is enhanced by formalin fixation. This particular antibody conjugate, therefore, is inadequate for the demonstration of antigen in tissues, although it might well have other uses. In progress is the preparation of conjugated antibodies in which it is expected fluorescence of a distinctive character will be secured.

*Summary.* A  $\beta$ -anthryl-carbamido derivative of antipneumococcus III rabbit antibody retains the original immunological properties while rendering Type III pneumococci specifically fluorescent in ultraviolet light.

It is a pleasure to acknowledge the constant advice and help so generously given by Dr. John F. Enders and Dr. Allan L. Grafflin, and the kind interest shown by Dr. Louis F. Fieser.

## 13085

### Effect of Local Application of Sulfanilamide upon Wound Healing.\*

MAX TAFFEL AND SAMUEL C. HARVEY.

*From the Department of Surgery, Yale University School of Medicine, New Haven, Conn.*

The efficacy of the sulfonamides in the systemic treatment of certain types of infection has been definitely established. During the past few years several communications<sup>1-6</sup> have described and recom-

\* Aided by a grant from the Fluid Research Funds of Yale University School of Medicine.

<sup>1</sup> Jaeger, K. H., *Deutsche med. Wchnschr.*, 1936, **62**, 1831.

<sup>2</sup> Jensen, N. K., Johnsrud, L. W., and Nelson, M. C., *Surgery*, 1939, **6**, 1.



mended the application of these drugs directly into raw wounds as a prophylactic or therapeutic measure against infection. Most of these reports based their contentions upon qualitative impressions gathered from the observation of a small series of patients rather than upon controlled studies. Before indiscriminately depositing these drugs into a raw wound it is essential to determine first of all whether they have any noxious effect upon the tissues involved in the healing of the wound. Bricker and Graham<sup>7</sup> reported that the systemic administration of sulfanilamide had an inhibitory effect upon the healing of stomach wounds in dogs during the first 7 postoperative days. Taffel and Harvey<sup>8</sup> found that sulfanilamide did not affect the healing of stomach wounds in rats during all of the normal phases of healing. The drug was given orally in doses sufficient to maintain an adequate and sustained "therapeutic" blood level.

This series of experiments was carried out to determine the effect of the local application of sulfanilamide upon the healing of a soft tissue wound. Adult white rats weighing about 250 g were used. The tensile strength of the wound served as an index of healing. In addition, histological studies were made of the wounds.

I. *Experimental Group.* 57 animals. This group was maintained throughout the duration of the experiment on a diet of Purina Dog Chow. On the 3rd day of the diet the stomach was delivered through an incision in the anterior abdominal wall and 0.2 cc of a 3% suspension of sulfanilamide in normal saline were injected through a fine needle into the wall of the cardia. This raised an edematous wheal which measured about 1.5 cm in diameter and which involved all the layers of the stomach wall. The finely powdered crystals of the drug were clearly visualized and appeared to be evenly distributed throughout the edematous zone. A wound was then made through one diameter of the wheal directly into the lumen of the stomach. The wound edges, finely stippled with drug particles, were immediately resutured in one layer with a running continuous Connell suture of No. 000 plain catgut, which, as has been previously shown, loses its tensile strength well within the 4th day. The abdominal wall was closed with 2 layers of fine No.

<sup>3</sup> Campbell, W. C., and Smith, H., *J. Bone and Joint Surg.*, 1940, **22**, 959.

<sup>4</sup> Herrell, W. E., and Brown, A. E., *Proc. Staff Meet., Mayo Clinic*, 1940, **15**, 611.

<sup>5</sup> Key, J. A., and Frankel, C. J., *Ann. Surg.*, 1941, **113**, 284.

<sup>6</sup> Rosenberg, S., and Wall, N. M., *Surg., Gynec. and Obst.*, 1941, **72**, 568.

<sup>7</sup> Bricker, E. M., and Graham, E. A., *J. A. M. A.*, 1939, **112**, 2593.

<sup>8</sup> Taffel, M., and Harvey, S. C., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 647.

A-silk, and 0.2 cc of normal saline were injected into the subcutaneous tissues of the groin. On each of the 4th, 6th, 8th, 10th, 12th, and 14th postoperative days 5 to 9 animals were sacrificed, and the strength of the wound immediately determined by distending the stomach with air and noting its bursting point. At each time interval the stomachs of 2 or more animals were reserved intact for histological examination of the wounds. No studies were made during the first 4 postoperative days, since it has been shown that during this interval the wound has only the strength contributed by the holding power of the suture.

II. *Control Group.* 54 animals. This group was similarly maintained upon a diet of Purina Dog Chow. On the 3rd day 0.2 cc of normal saline were injected into the wall of the cardiac end of the stomach, and wounds similar to those of the experimental group were made. At the close of the operation 0.2 cc of a 3% suspension of sulfanilamide in normal saline were injected into the subcutaneous tissues of the groin. Tensile strength and histological studies were carried out from the 4th through the 14th postoperative day.

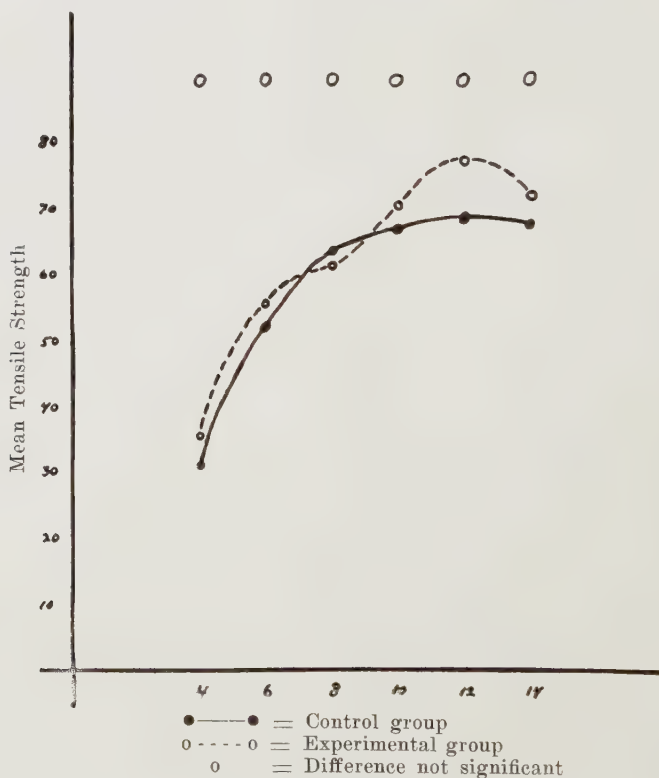


FIG. 1.



The stomach wounds all healed *per primam*. In each group the averages of the breaking strength and the standard deviation for every postoperative interval were computed. Fisher's<sup>9</sup> formula for small samples was applied to determine whether the differences in the means of the two groups were statistically significant.

*Results.* The local application of sulfanilamide crystals in the edges and in the immediate vicinity of stomach wounds in rats did not affect the healing of these wounds. (Fig. 1.) Histological studies of the wounds in the experimental group revealed the rare presence of the sulfanilamide crystals. These were engulfed within multinucleated giant cells which were located only in the serous and submucous coats at the margins of the wound, but not in the zone of actively proliferating fibroblasts. Except for this mild foreign body reaction the microscopic picture was essentially the same as that in the control group.

### 13086 P

#### Functional Localization Within the Anterior Cerebellum.

GERVASE J. CONNOR. (Introduced by S. C. Harvey.)

*From the Departments of Surgery and Physiology, Yale University School of Medicine, New Haven, Conn.*

The anterior cerebellar lobe is that portion of the corpus cerebelli lying anterior to the primary fissure. It includes 3 major subdivisions, the culmen, centralis and lingula; the first 2 of these areas possess definite hemispherical expansions. In a previous communication<sup>1</sup> a short description was given of the postural deviations which follow removal of the entire anterior cerebellar lobe. The present report deals with the results of further studies in the dog, cat and monkey, designed to investigate the problem of localization of function within this region.

The ablations were made from above through the tentorium. The series included 53 dogs, 18 cats and 23 monkeys. The animals were observed postoperatively for periods ranging from 5 days to 1½ years, were sacrificed under anesthesia, the brains removed and studied grossly. Detailed histological studies were obtained in 42 animals; appropriate serial sections of the brain stem and cerebel-

<sup>9</sup> Fisher, R. A., *Statistical Methods for Research Workers*, Edinburgh, 1934.

<sup>1</sup> Fulton, J. F., and Connor, G. J., *Trans. Am. Neurol. Soc.*, 1939, 53.

lum in each were stained by either the Spielmeyer, Kulschitsky, Nissl or hemotoxylin and eosin method. The results may be summarized as follows:

I. *The spinal (paleocerebellar) anterior lobe.*

(1) *Ablation of the culmen* alone provokes a complex of signs entirely similar in direction to those already described in the total anterior decerebellate preparation, but, strikingly, they are limited to the hindlegs, each of which is equally involved. The forelegs and the neck are unaffected. The syndrome of this region is characterized by a profound release of the antigravity muscles in the hindlegs, of such a nature that these muscles respond hyperactively to all extensor postural influences. The local static reflexes are conspicuously increased; thus, the positive supporting reaction (Stütz response) is strongly hyperactive and well-defined lengthening and shortening reactions are present. The segmental static responses are similarly released; the reflexes of stance, the Schunkel, Hinkebein and Stemmbein reactions are increased to such a degree that, in the hindlegs, the gait is crooked and weaving, the extremities exhibit marked errors in range, rate, force and direction and are placed inefficiently for smooth balance. Moreover, the strength of these postural responses is distinctly modified by general static influences; thus, the neck tonic and labyrinthine tonic influences result in a further increase or decrease, appropriately, in each of these reactions. The deep tendon reflexes of the involved hindlegs are likewise hyperactive and spreading and can be modified by changes in the animal's position in space. This extensor release is significantly less in degree than that observed after total anterior decerebellation, for reasons which will be noted.

Further restriction of the ablation to one lateral expansion of the culmen results in entirely similar signs but with their further *limitation to the ipsilateral hindleg alone*. There thus appears a complex of signs restricted solely to a *single extremity*.

(2) *The posterior centralis*, which possesses a definite hemispherical expansion, controls, similarly, the forelegs alone. Extirpation restricted to this region is followed by the signs already noted but they are now limited to both forelegs, each of which is equally involved. Further restriction of this ablation to one hemispherical expansion in this region provokes the same signs but, again, these are limited to a *single extremity*, the ipsilateral foreleg.

(3) *The anterior centralis*, a predominantly midline structure, presides in like manner over the neck extensors. The signs are limited to the neck and are somewhat more subtle than those already



noted. There appears an increased extensor postural sensitivity in the neck muscles but a conspicuous opisthotonos fails to appear unless the "released" neck muscles are further reflexly stimulated (see below).

II. *The vestibular (lingula) anterior lobe.* (4) Following pure lesions of the lingula the signs are such as to suggest "release" of the labyrinthine tonic influences. Though disequilibrium is profound, local and segmental postural reflexes in the extremities are entirely normal provided support is afforded the animal's head. The labyrinthine tonic effects upon the extremities are, however, abnormally increased. The syndrome fails to appear after preliminary labyrinthectomy.

(5) Results of combinations of these discrete ablations indicate that opisthotonos appears only when the neck extensors are released and, in addition, a tonic influence upon them is also increased above normal. Thus, the prime requisite is that the anterior centralis be removed; then, either the labyrinthine tonic influences must be released by lingular ablation (resulting, compositely, in an extreme opisthotonos), or the foreleg extensor influence upon the neck be released by posterior centralis removal (resulting, compositely, in a mild opisthotonos).

*Summary.* Ablation studies in the dog, cat and monkey reveal a functional type of localization within the anterior cerebellar lobe. This localization is so precise that functional units in single extremities, the neck and the labyrinths are discretely represented in isolated anterior cerebellar subdivisions.

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**Developmental Rate of Hybrids between *Rana pipiens* and  
*Rana sphenocephala*.**

JOHN A. MOORE. (Introduced by L. G. Barth.)

*From the Department of Biology, Brooklyn College, Brooklyn, N.Y.*

This forms the second report on developmental rate in hybrid frogs. The purpose of these experiments, in hybridizing species that differ in rate of development, is to determine the earliest effect of the sperm on this particular character. Previously such a study has been made on hybrids between *Rana pipiens* and *Rana palustris*.<sup>1</sup>

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<sup>1</sup> Moore, *J. Exp. Zool.*, 1941, **86**, 405.

Both hybrids, *pipiens* ♀ X *palustris* ♂ and *palustris* ♀ X *pipiens* ♂ developed at the maternal rate during early stages. The first unmistakable deviation from the maternal rate appeared in the neural plate stage in both hybrids. Additional evidence on these points is derived from the following crosses.

The material used in these experiments is from the following sources: *Rana pipiens* Schreber from dealers in the Lake Champlain area of Vermont and *Rana sphenocephala* (Cope) from dealers in southern Florida. Although listed as separate species by many taxonomists the two are closely related and are thought best included under one name by Kellogg.<sup>2</sup> Regardless of their true taxonomic status the two frogs form viable hybrids and differ in rate of growth to such an extent that it is possible to analyze the developmental rate of their hybrids. At 15°C the rate of both species is identical. At higher temperatures *sphenocephala* develops more rapidly and below 15° less rapidly than *pipiens*. The egg diameter in the *sphenocephala* used in these experiments averaged 1.3 mm and in the *pipiens* 1.8 mm. Whereas the upper limit for normal development in *pipiens* is 28° or 29°, the Florida species can tolerate temperatures as high as 34.5°.

The first experiment was begun Jan. 20, 1939. Gametes of both male and female *pipiens* and *sphenocephala* were used, the eggs being secured by pituitary injections and sperm by cutting the testes in 0.1 amphibian Ringer's. One hour before first cleavage the 2 groups of control eggs (*pip.* ♀ X *pip.* ♂ and *sph.* ♀ X *sph.* ♂) and the 2 groups of hybrids (*pip.* ♀ X *sph.* ♂ and *sph.* ♀ X *pip.* ♂) were placed in an incubator which maintained water temperature at  $19.6 \pm 0.1^\circ\text{C}$ . Because of the difference in size between the eggs of the 2 species it was possible to keep both the *sphenocephala* and *pipiens* controls in one bowl and the *pip.* ♀ X *sph.* ♂ and *sph.* ♀ X *pip.* ♂ hybrids together in another. This reduced the error of measuring rate of development due to temperature variations.

The interval between cleavages was not measured in this experiment. The *sphenocephala* controls and *sph.* ♀ X *pip.* ♂ began gastrulation (stage 10<sup>3</sup>) 17½ hours after first cleavage (Table I). At 20 hours the *sphenocephala* controls were in stage 11. In the *sph.* ♀ X *pip.* ♂ gastrulation seemed not to have progressed quite so far. At 24 hours the *pipiens* controls and *pip.* ♀ X *sph.* ♂ had begun gastrulation. By this time the *sphenocephala* controls had a complete blastopore (stage 12). The *sph.* ♀ X *pip.* ♂ appeared somewhat

<sup>2</sup> Kellogg, *Bull. U. S. National Museum*, 1932, 160.

<sup>3</sup> Pollister and Moore, *Anat. Rec.*, 1937, **68**, 489.



retarded at this time, as they were at 20 hours. However, this difference was slight and may not have been of significance. At 28½ hours after first cleavage an unmistakable retardation in rate was noticed in the *sph.* ♀ X *pip.* ♂. The *sphenocephala* controls at this time were in stage 13 (neural plate) whereas the hybrids in question were still in stage 12. The reciprocal hybrids showed the first unmistakable deviation from the maternal rate at about the same morphological stage. Forty-five and one-half hours after fertilization when the *pipiens* controls were in stage 14 (neural folds) the *pip.* ♀ X *sph.* ♂ were still in stage 13. Previous to this time it was not possible to detect any difference in rate between these hybrids and the maternal controls.

When examined at later times it was found that both groups of hybrids continued to be slower in development than their respective controls. The *sph.* ♀ X *pip.* ♂ were the most retarded group and exhibited a rather constant morphological deviation, the heads of these tadpoles being very small. In the reciprocal cross, *pip.* ♀ X *sph.* ♂ the heads seemed to be much too large.

Some of these animals together with others from a similar experiment were placed in tanks and many hybrids of both types were raised to large tadpoles and young frogs. In early stages when the different groups were examined carefully no difference in mortality could be noticed. In all groups about 95% gave rise to normal tadpoles.

Further experiments were undertaken in the 1940 season to study the rate of cleavage in the hybrids and to pay special attention to the stage at which hybrids deviated from the maternal rate of develop-

TABLE I.  
Development of *Rana pipiens*, *Rana sphenocephala*, and their hybrids at  
19.6 ± 0.1°C.

Hrs. after 1st cleavage	Stage of development			
	<i>sphenocephala</i> controls	<i>sph.</i> ♀ X <i>pip.</i> ♂ hybrids	<i>pip.</i> ♀ X <i>sph.</i> ♂ hybrids	<i>pipiens</i> controls
17½	10	10	9	9
20	11	10	9	9
24	12	12 (early)	10	10
26	12	12	10	10
28½	13	12	11	11
31	13	13	12	12
37	14	13	12	12
45½	14	14	13	14
48½	16	15	14	14
54½	16	16	14	16
67	17	17	16	17
76½	18	18	17	18
89	20	18	18	18
102½	20	19	19	20

ment. In these experiments the only hybrids studied were *pip.* ♀ X *sph.* ♂.

No deviation from the maternal rate of cleavage was noticed. At  $23.8 \pm 0.1^{\circ}\text{C}$  the *pipiens* controls reached first cleavage in 112 minutes after fertilization. The time for the *pip.* ♀ X *sph.* ♂ was 113 minutes. The interval between first and second cleavage was 40 minutes for both groups of eggs. The *pipiens* controls required 39 minutes between second and third cleavage and the *pip.* ♀ X *sph.* ♂ 40 minutes.

Three experiments were carried out at  $23.8 \pm 0.1^{\circ}\text{C}$  to detect the first deviation of the *pip.* ♀ X *sph.* ♂ hybrids from the maternal rate. In the first the hybrids were possibly retarded at 20 hours after first cleavage (stage 12). In the second experiment a definite retardation was noticed at  $23\frac{3}{4}$  hours (stage 12). In the third experiment the hybrids and controls were identical at 18 and 21 hours (stage 12). In all cases the retardation was unmistakable during neural plate stages. Experiments at  $27.5^{\circ}$ ,  $18.5^{\circ}$ , and  $15.5^{\circ}$  gave similar results. In all experiments there seemed some indication that the retardation in rate of development began near the close of gastrulation. This retardation was invariably evident in neural plate stages.

In contrast with the regular development of the hybrids in the 1939 season the mortality and percentage of abnormal tadpoles during 1940 was extremely high. The most obvious external abnormality of the *pip.* ♀ X *sph.* ♂ hybrids was in the functioning of the circulatory system. Gill circulation was abnormal or lacking in most embryos. The gill filaments were usually bulbous at the end and the presence of a large bleb at the base of the gills seemed characteristic. In many the heart was not beating. Over 95% of these embryos died as young tadpoles.

At the present no explanation of the difference in results obtained the two seasons is offered. The collector who supplied the animals states that during 1940 lack of rain and cold had been very hard on the frogs and they were not "in breeding condition." The cause of these abnormalities, interesting in itself, is apart from the main purpose of the experiments. In all the observations on hybrid rate of development are consistent. The developmental rate of the hybrids is maternal until late in gastrulation. Possibly towards the end of gastrulation and certainly in the neural plate stage the sperm exerts an effect on the rate of development. This is precisely the stage at which the first unmistakable effect of the sperm was noticed in the *pipiens* X *palustris* cross mentioned before.<sup>1</sup>

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## Serum Protection Tests with the Lansing Strain of Murine Poliomyelitis Virus.

C. G. HARFORD AND J. BRONFENBRENNER.

*From the Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis, Mo.*

Armstrong's<sup>1, 2</sup> method for the adaptation of the Lansing strain of human poliomyelitis virus has consisted of preliminary passage in monkeys and cotton rats. Because previous attempts by many workers<sup>3</sup> to adapt the virus of poliomyelitis by direct passage to mice have failed, it has been suggested that the Lansing virus in mice may possibly represent not the original human virus but some other virus picked up during that passage. Although Armstrong<sup>2</sup> showed that the sera of some monkeys convalescent from the disease protected against the virus, we thought that further study of the identity of the virus by means of serum protection tests was desirable.

In our experiments protection tests were carried out in such a way as to allow direct statistical analysis of the findings and have found that serum tests carried out and evaluated as described give significant results. The protection tests were carried out mainly with human and monkey sera taken early and late after onset of paralysis in order to see whether antibodies capable of protecting against the Lansing strain developed following the paralytic disease. We have included also a sample of pooled human convalescent serum as well as the serum of rabbits immunized by injections of mouse passage virus.

Fresh virus suspensions were prepared for each individual experiment by grinding the base of the brain and the spinal cord of one or two paralyzed mice with enough saline to make about a 1:4 suspension. Only mice paralyzed within 8 days after inoculation and those in which paralysis had existed for not longer than 2 days were used. After centrifugation, 0.75 cc of the supernatant was added to an equal amount of serum, mixed thoroughly and without further incubation 0.03 cc of the mixture was injected intracerebrally into each of 20 Swiss mice. Care was taken to make the control normal

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<sup>1</sup> Armstrong, C., *Pub. Health Rep.*, 1939, **54**, 1719.

<sup>2</sup> Armstrong, C., *Pub. Health Rep.*, 1939, **54**, 2303.

<sup>3</sup> Harmon, P. J., Shaughnessy, J. H., and Gordon, F. B., *J. Prev. Med.*, 1930, **4**, 59, 89.



Experiment No.	Age of mice (days)
1	10
2	15
3	20
4	25
5	30
6	35
7	40
8	45
9	50
10	55
11	60
12	65
13	70
14	75
15	80
16	85
17	90
18	95
19	100
20	105
21	110
22	115
23	120
24	125
25	130
26	135
27	140
28	145
29	150
30	155
31	160
32	165
33	170
34	175
35	180
36	185
37	190
38	195
39	200
40	205
41	210
42	215
43	220
44	225
45	230
46	235
47	240
48	245
49	250
50	255
51	260
52	265
53	270
54	275
55	280
56	285
57	290
58	295
59	300
60	305
61	310
62	315
63	320
64	325
65	330
66	335
67	340
68	345
69	350
70	355
71	360
72	365
73	370
74	375
75	380
76	385
77	390
78	395
79	400
80	405
81	410
82	415
83	420
84	425
85	430
86	435
87	440
88	445
89	450
90	455
91	460
92	465
93	470
94	475
95	480
96	485
97	490
98	495
99	500
100	505
101	510
102	515
103	520
104	525
105	530
106	535
107	540
108	545
109	550
110	555
111	560
112	565
113	570
114	575
115	580
116	585
117	590
118	595
119	600
120	605
121	610
122	615
123	620
124	625
125	630
126	635
127	640
128	645
129	650
130	655
131	660
132	665
133	670
134	675
135	680
136	685
137	690
138	695
139	700
140	705
141	710
142	715
143	720
144	725
145	730
146	735
147	740
148	745
149	750
150	755
151	760
152	765
153	770
154	775
155	780
156	785
157	790
158	795
159	800
160	805
161	810
162	815
163	820
164	825
165	830
166	835
167	840

\*The figures indicate the day after inoculation that paralysis was first observed. Italicized figures indicate the day of death, during the night, without paralysis having been observed previously.  
†In most instances all animals were of exactly the same age (indicated in days). Where the exact age is not indicated, animals of the same weight were used.  
‡These data were used as control in calculations.  
§Numerator indicates the number infected; denominator the total number of animals.  
||The immune rabbit serum was obtained as follows: Each of 2 rabbits was given a total of 12 injections of half of a suspension prepared by grinding a glycerinated spinal cord and base of the brain with 2 cc of saline. Rabbits were bled 2 weeks later.  
Sign “D” indicates that the mouse died of a cause known not to be poliomyelitis; such ones are not included in the evaluation of the result.  
A chi square value over 3.841 is considered “significant,” and one over 6.635 is considered “highly significant.”

<sup>†</sup>In most instances all animals were of exactly the same age (indicated in days). Where the exact age is not indicated, animals of the same weight paralysis having been observed previously, were used.

† These data were used as control in calculations.

Myelinated spinal cord and base of the brain with 2 cc of saline. Rabbits were bled 2 weeks later.

lycerinated spinal cord and base of the brain with 2 cc of saline. Rabbits were bled 2 weeks later.

Sign "D" indicates that the mouse died of a cause known not to be polio myelitis; such ones are included in the evaluation of the result. A chi square value over 3.841 is considered "significant," and one over 6.635 is considered "highly significant."

serum-virus mixtures first and to inject the immune serum-virus mixtures first so that variation in time of contact with serum or time before injection could not influence the result.

The incubation period with this infection has varied within wide limits (2 days to 59 days) and, in spite of about 30 passages, this variability still persists. For this reason, in each case we have recorded the number of days elapsed between the injection and the first manifestation of infection. Calculation from the figures in Table I shows that, of those animals eventually dying or becoming paralyzed, 24% did so after an incubation period of 21 days. Furthermore, it will be noted that a greater proportion of long incubation periods tended to occur in mice receiving injections of immune serum-virus mixtures.

Schaeffer and Muckenfuss<sup>4</sup> have emphasized the irregularities in the results of protection tests encountered when the monkey is used as a test animal. This appears to be true also with respect to results obtained with tests in mice. However, calculation of the value of chi square, as described by Mainland,<sup>5</sup> indicates that our results represent data which are statistically valid. We believe that, following the procedure described above, we have avoided the use of more animals than necessary and also have obtained results of maximum significance.

The sample of pooled human convalescent serum used in Experiment 1 was part of that which was subjected to repeated testing in monkeys by Schaeffer and Muckenfuss. In our single test with the Lansing strain in mice, it also gave protection.

Neither of the samples of serum of the patient R.T. neutralized the virus (Experiment 2). This boy had definite clinical paralytic disease and may have developed protective antibody, but the failure of his serum to neutralize the Lansing virus might have been due to the fact that his infection was caused by an antigenically heterogeneous strain.

Both of the samples of J. M. showed the presence of antiviral property and this is thought to be due to the fact that the first sample was taken so late after the acute stage of the infection (17 days) that the development of antibody had already taken place (Experiment 3).

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<sup>4</sup> Schaeffer, M., and Muckenfuss, R. S., *Experimental Poliomyelitis*, published under the auspices of the National Foundation for Infantile Paralysis, 1940.

<sup>5</sup> Mainland, D., *The Treatment of Clinical and Laboratory Data. An Introduction to Statistical Ideas and Methods for Medical and Dental Workers*, Oliver and Boyd, 1938.

We have no explanation as to why the serum of E.H. showed antibody shortly after onset of paralysis but failed to protect mice later on (Experiment 4).

Sera of Monkey R.H. 42 were obtained from Dr. A. B. Sabin. This animal had been paralyzed by the MV strain of poliomyelitis virus and protection tests carried out in monkeys by him had yielded the same results as those we have secured by protection tests in mice (Experiments 5, 6, and 7). The main reason for repeating the experiments with monkey sera is the fact that one presumably normal monkey serum (Experiment 5) appeared to have exhibited a considerable amount of protection. These experiments with the monkey sera indicate that antibody appeared in this animal between one and 3 months after paralysis.

Although the injection with the serum of immunized rabbits resulted in fewer animals developing the disease, calculation showed that results of this experiment were not statistically significant (Experiment 8).

Thus, while some of the human sera neutralized with Lansing virus, we have been unable so far to secure evidence that such neutralizing antibodies appear shortly after the clinical signs of infection. Similar findings with monkey tests have been obtained by other workers.<sup>6, 7</sup> However, the experiments with the sera of the monkey definitely indicate the development of neutralizing antibody after paralysis. These limited experiments cannot be said to have proved the identity of the Lansing virus. However, both the results with human and with monkey sera are consistent with the supposition of the existence of a serological relationship between the Lansing strain and the virus causing some cases of human disease.

Since we began these experiments, the reports of serum protection tests by Haas and Armstrong,<sup>8</sup> and with similar strains by Jungeblut and Sanders<sup>9</sup> and Toomey and Takacs<sup>10</sup> have been published. Nevertheless, we have considered our results worthy of recording not only because they strengthen the evidence in favor of considering the virus of Armstrong as immunologically related to poliomyelitis, but

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<sup>6</sup> Brodie, M., Fischer, A. E., and Stillerman, M., *J. Clin. Invest.*, 1937, **16**, 447.

<sup>7</sup> Burnet, F. M., and Jackson, A. V., *Australian J. Exp. Biol. and Med. Sci.*, 1939, **17**, 261.

<sup>8</sup> Haas, V. H., and Armstrong, C., *Pub. Health Rep.*, 1940, **55**, 1061.

<sup>9</sup> Jungeblut, C. W., and Sanders, M., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **44**, 375; *J. Exp. Med.*, 1940, **72**, 407.

<sup>10</sup> Toomey, J. A., and Takacs, W. S., *PROC. SOC. EXP. BIOL. AND MED.*, 1941, **46**, 319.



also because they have been considered in connection with the time of onset of paralysis. This has seemed particularly desirable since Haas and Armstrong found that the occurrence of antibody could be correlated more closely with age than with the clinical disease.

*Summary.* Serum protection tests in mice have been done and the results are significant as tested by a simple statistical method. The experiments are in favor of the view that the Lansing strain is immunologically related to human poliomyelitis.

We acknowledge, with thanks, assistance from the following: Mr. Louis Mihelich and Miss Frances Love (technical assistance), and Doctor Martin Bronfenbrenner (advice on statistical method).

### 13089

#### A Human Serum Containing Four Distinct Isoagglutinins.

ALEXANDER S. WIENER\* AND SAMUEL FORER. (With the technical assistance of Eve Sonn.)

*From the Serological Laboratory, Office of the Chief Medical Examiner, New York City.*

The serum to be described was studied because the patient from whom it was derived had had hemolytic reactions following transfusions of apparently compatible blood. In this report we shall present the serologic findings; the clinical data will be described elsewhere.<sup>1</sup>

The patient belonged to group O, type N. The patient's serum agglutinated every one of 28 consecutive group O bloods. That we were not dealing with an autoagglutinin was proved by the absence of agglutination in mixtures of the patient's serum with her own cells. Evidently the serum contained isoagglutinins in addition to anti-A and anti-B.

Since the patient was Rh negative,<sup>2, 3</sup> it seemed possible that anti-Rh isoagglutinins might be present in her serum. Accordingly the serum was absorbed with pooled, washed cells A<sub>1</sub>MNRh- and

\* Aided by a grant from the Committee on Human Heredity of the National Research Council.

<sup>1</sup> Wiener, A. S., Forer, S., and Crooks, P. E., in preparation.

<sup>2</sup> Landsteiner, K., and Wiener, A. S., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 223.

<sup>3</sup> Wiener, A. S., and Peters, H. R., *Ann. Int. Med.*, 1940, **13**, 2306.

BMNRh-. The absorbed serum was found to give reactions coinciding with those of standard Rh antisera, proving that the anti-Rh isoagglutinin was present. Since tests on Rh negative bloods with the unabsorbed serum showed that most of the bloods were agglutinated, it was evident that another irregular isoagglutinin was present. Difficulty was encountered in separating the unknown isoagglutinin from the anti-Rh isoagglutinin by absorption experiments. A separation between them was readily effected, however, when it was found that the anti-Rh isoagglutinin acted strongly at body temperature, and not at all at refrigerator temperature, while the unidentified isoagglutinin acted only in the cold.

In order to ascertain the nature of the fourth isoagglutinin, it was necessary to test a larger series of bloods. On account of the presence in the patient's serum of anti-A and anti-B isoagglutinins, bloods of groups A, B, and AB could not be tested directly. It was found that by the simple addition to the patient's serum of a solution of the group substances A and B (Table I), the action of the interfering  $\alpha$  and  $\beta$  isoagglutinins could be completely neutralized without affecting the activity of the anti-Rh isoagglutinin or the other irregular isoagglutinin. Moreover, by making tests with serial dilutions of the patient's serum at body and refrigerator temperatures, the reactions of the anti-Rh isoagglutinin and the fourth isoagglutinin were readily separated as shown in Table I. It will be seen that every blood not clumped by the patient's serum at low temperature, belongs to type N, like that of the patient, suggesting that the fourth isoagglutinin is an anti-M isoagglutinin. That this is correct was established by tests on a series of bloods including 10 of type N.

From the serologic standpoint, these observations are of some interest. Firstly, this is apparently the first human serum to be described in which 2 different irregular isoagglutinins could be clearly identified. Secondly, this is one of the few human sera to be described with anti-M isoagglutinins in it, of which only three<sup>4, 5, 6</sup> have previously been encountered among many thousands of human sera examined. The serum is also unusual because of the high titer of the anti-Rh isoagglutinin, the titer being 64 at 37°C. It is interesting that despite the high titer, the anti-Rh isoagglutinins were not active at all at ice-box temperature, in contrast to the sera previously reported by Wiener and Peters.<sup>3</sup> Such anti-Rh isoagglutinins acting at body temperature have been described by Levine, Katzin and

<sup>4</sup> Wolff, E., and Jonsson, B., *Deutsch Z. f. d. ges. gerichtl. Med.*, 1933, **22**, 84.

<sup>5</sup> Friedenreich, V. *Z. f. Immunitäts.*, 1937, **91**, 485.

<sup>6</sup> Moureau, P., and Lambert, J., *Ann. de Med. Leg.*, 1940, **20**, 163.

TABLE I.  
Reactions of Serum 1 of Patient on Series 2 of 17 Bloods.

Blood specimen No.	Group and/or subgroup type	Bloods tested <sup>3</sup> for Rh factor at 37°C with patient's serum dilution						Interpretation of reactions with patient's serum at body temp.	Results of tests with Anti-Rh Immune Serum <sup>1</sup>	Bloods tested <sup>5</sup> for M factor in refrigerator with patient's serum dilution		Patient's serum absorbed with bloods 1-17 and tested vs. blood 14 <sup>6</sup>	Interpretation of reactions with patient's serum at 0°C
		1:4	1:8	1:16	1:32	1:64	1:128			1:1	1:2		
1	B	++	++	++	++	tr	—	Pos.	Pos.	++	+±	—	Pos.
2	B	++	++	++	++	+±	tr	Pos.	Pos.	++	+	—	Pos.
3	A <sub>2</sub>	++	++	++	++	tr	—	Pos.	Pos.	++	+	—	Pos.
4	A <sub>1</sub>	++	++	++	++	tr	—	Pos.	Pos.	++	+±	—	Pos.
5	B	++	++	++	++	+	—	Pos.	Pos.	++	+±	—	Pos.
6	A <sub>1</sub> B	tr.	—	—	—	—	—	Neg.	Neg.	++	+	—	Pos.
7	A <sub>1</sub>	++	++	+±	+	+	tr	Pos.	Pos.	++	+	—	Pos.
8	A <sub>1</sub>	++	++	+±	+±	tr	—	Pos.	Pos.	++	+±	—	Pos.
9	A <sub>2</sub>	Hem.	++	+±	+	tr	—	Pos.	Pos.	++	+±	—	Pos.
10	O	Hem.	++	+±	+±	tr	—	Pos.	Pos.	++	+±	—	Pos.
11	A <sub>1</sub>	+	+	+	+±	tr	—	?	Pos.	++	+±	—	Pos.
12	O	++	++	+±	+±	+	—	Pos.	Pos.	++	+	—	Neg.
13	B	++	++	+	+	+	—	Pos.	Pos.	++	+	—	Pos.
14	O	++	++	+	+	+	—	Pos.	Pos.	++	+	—	Pos.
15	A <sub>1</sub> B	—	—	—	—	—	—	Neg.	Neg.	++	+	—	Neg.
16	O	++	++	++	++	tr	—	Pos.	Pos.	++	+	—	Neg.
17	A <sub>1</sub> B	++	++	++	++	—	—	Neg.	Neg.	++	+±	—	Pos.
Donor	O	—	—	—	—	—	—	Neg.	Neg.	—	—	—	Neg.

<sup>1</sup> The patient's serum before use was prepared as follows. It was first inactivated by heating at 56°C for 20 minutes, and then it was mixed with one-fifth its volume of a "purified" solution of group substances A and B. The latter was prepared from salivas of group A and group B, heated in boiling water for 10 minutes, then centrifuged and cleared by filtration through a half thickness Seitz pad, the filtrates being pooled in the proportion of 2 parts A saliva to 1 part B saliva.

<sup>2</sup> The series tabulated is random except that individuals were selected so as to include representatives of each of the 4 blood groups. For comparison, the reactions of the blood of one of the compatible donors used for subsequent transfusions are also included.

<sup>3</sup> One drop each of serum dilution, blood suspension (2% in terms of blood sediment) and saline were mixed in small test tubes. Preliminary readings were taken after 1 hour at 37°C; final readings after standing over night at room temperature.

<sup>4</sup> These tests were made with anti-rhesus immune guinea-pig sera (cf. Landsteiner, K., and Wiener, A. S., article in preparation).

<sup>5</sup> One drop each of serum, blood suspension and saline were mixed in small test tubes, the rack was placed in the refrigerator, preliminary readings taken in 1 hour, final readings the next day.

<sup>6</sup> One drop of the packed, washed cells of each blood tested was mixed with 3 drops of the patient's serum. After 1 hour in the refrigerator, a drop of the supernatant was removed, tested against blood suspension No. 14, the latter blood being selected since it belonged to group O and was Rh negative, so that any agglutination obtained had to be due to the M factor.



Burnham,<sup>7</sup> however. Incidentally, the fact that it was possible to neutralize the interfering anti-A and anti-B isoagglutinins in human sera without affecting the irregular isoagglutinins, by the simple expedient of adding solutions of group substances from saliva, is evidence that the properties Rh and M are absent from saliva, in contrast to A and B.<sup>8</sup>

From the clinical standpoint, the case illustrates the difficulties that may be encountered on rare occasions in finding compatible donors for blood transfusion. That three subsequent transfusions from two different selected donors of type ONRh- were successful indicates that such obstacles can be overcome.

The authors wish to thank Dr. Harry Aranow, Director of Obstetrics, and Dr. P. E. Crooks, Resident in Obstetrics and Gynecology, of the Morrisania City Hospital for their coöperation in providing samples of the patient's blood.

## 13090

### Adrenolytic Action of Cyclopropane.\*

J. W. STUTZMAN AND C. R. ALLEN. (Introduced by W. J. Meek.)

*From the Department of Physiology, University of Wisconsin, Madison.*

Previous work<sup>1, 2, 3</sup> has shown that 30 minutes of cyclopropane anesthesia sensitizes the heart of the dog so that ventricular tachycardia follows the injection of 0.01 mg of adrenalin<sup>†</sup> per kilogram. The only ventricular effect of this dose in the unanesthetized animal is extrasystoles. In studying the mechanism of this sensitization it was observed that some dogs which showed 30 seconds or more of

<sup>7</sup> Levine, P., Katzin, E. M., and Burnham, L., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **45**, 346.

<sup>8</sup> Dr. Philip Levine (personal communication) has also found that the property Rh is probably restricted to the blood cells.

\* Supported in part by grants from E. R. Squibb & Sons and the Wisconsin Alumni Research Foundation.

<sup>1</sup> Meek, W. J., Hathaway, H. R., and Orth, O. S., *J. Pharm. and Exp. Therap.*, 1937, **61**, 240.

<sup>2</sup> Orth, O. S., Leigh, M. D., Mellish, C. H., and Stutzman, J. W., *J. Pharm. and Exp. Therap.*, 1939, **67**, 1.

<sup>3</sup> Allen, C. R., Stutzman, J. W., and Meek, W. J., *Anesthesiology*, 1940, **1**, 158.

<sup>†</sup> Parke Davis adrenalin hydrochloride.

cyclopropane-adrenalin tachycardia after 30 minutes of anesthesia had no irregularities when adrenalin was injected subsequently after the anesthesia had continued for a longer time. This paper is an investigation of this adrenolytic action of cyclopropane on the cardiovascular system. By adrenolytic action is meant the blocking of the response of the effector organs to the injection of adrenalin. In this study blood pressure and cardiac responses were used as indicators.

In order to determine the percentage of dogs that develop this tolerance to adrenalin within 5 hours of cyclopropane anesthesia 31 dogs were anesthetized without premedication, intubated to insure an open airway, and connected through a soda-lime carbon

TABLE I.  
Duration of Ventricular Tachycardia in Dogs Following the Injection of 0.01 mg  
Adrenalin per Kilogram under Cyclopropane Anesthesia.

Dog No.	Minutes of cyclopropane anesthesia							Remarks
	5	30	90	120	150	210	300	
	sec	sec	sec	sec	sec	sec	sec	
1	12	0						Complete protection
2	30	0						
3		18	0					
4		28	0					
5		30	0					
6		40	0					
7		44	0					
8		55	0					
9		65			0			
10		37	48		31	0		
11		65		40		0		
12		70	72		64	0		
13		71	45		17	0		
14		29	40		44	35	0	
15		32	45		30	30	0	
16		52	25		30		0	
17		65	30		30	25	0	
18		76	63		63		0	
19		79		73		66	0	
20		79	66		38	36	15	Partial protection
21		76	72		35	25	18	
22		70	68		65	70	20	
23		60	50	50	50	50	30	
24		60	70		45	47	40	
25		76	67			64	51	
26		51	51	35	42	41	38	No protection
27		50	49		45	44	43	
28		48	64		61	54	73	
29	Ventricular fibrillation							
30		"		"				
31		"		"				

dioxide absorber to a 100 liter bag containing a 32% mixture of cyclopropane in oxygen. In dogs this concentration of the anesthetic agent produces deep surgical anesthesia with at least partial intercostal paralysis. After 30 minutes of this constant mixture the dogs were injected intravenously with 0.01 mg of adrenalin per kilogram in 5 cc of normal saline at a constant rate of 1 cc per 10 seconds. The duration of ventricular tachycardia was determined by electrocardiograms (lead II) taken throughout a 5-minute period beginning with the injection. The injection of adrenalin was repeated after 90, 150, 210, and 300 minutes of cyclopropane anesthesia or until no ventricular tachycardia occurred. Direct blood pressure tracings were made of 9 of the dogs.

From Table I it can be seen that 19 of the 31 dogs developed a complete protection to cyclopropane-adrenalin tachycardia in 300 minutes or less of anesthesia. Of these animals 2 had no ventricular tachycardia after 30 minutes; 6 after 90 minutes; 1 after 150 minutes; 4 after 210 minutes; and 6 after 300 minutes. Of the remaining 12 dogs 6 had an average ventricular tachycardia of 70 seconds' duration when adrenalin was injected after 30 minutes of anesthesia but had an average of only 29 seconds tachycardia following the injection after 300 minutes. These dogs were considered as being partially protected. Three of the remaining dogs showed no protection within 300 minutes of anesthesia and 3 died of ventricular fibrillation as a result of the first injection of adrenalin.

After the development of complete protection higher doses of adrenalin were given to 13 dogs. Table II indicates the results. Each of the 10 dogs receiving 2 times the control dose showed no tachycardia. Three of 9 dogs had ventricular tachycardia following the injection of 5 to 10 times the control dose. There was no case of ventricular fibrillation with high doses of adrenalin even though 4 of the animals received 1 mg per kilogram, which is 100 times the control dose and more than the M.L.D. for the unanesthetized dog.<sup>4</sup> The degree of protection appears to be of the same order as that produced by decerebration, by bilateral sympathectomy,<sup>3</sup> by blocking the sympathetics with sympatholytic agents, or by protecting the heart with adrenolytic drugs.<sup>5</sup>

The blood pressure levels and the responses to the injections of adrenalin before and after complete protection from cyclopropane-adrenalin tachycardia are listed in Table III. The average pressure

<sup>4</sup> Parkins, W. M., Swingle, W. W., Taylor, A. R., and Hays, H. W., *Am. J. Physiol.*, 1938, **123**, 669.

<sup>5</sup> Orth, O. S., and Allen, C. R., *Am. J. Physiol.*, in press.



TABLE II.  
Increased Doses of Adrenalin under Cyclopropane Anesthesia.  
Duration of Ventricular Tachycardia.

Dog No.-	Before adrenolytic action	After adrenolytic action					Time for adrenolytic action to occur min
	Control dose sec	Same dose sec	2x's dose sec	5x's dose sec	10x's dose sec	100x's dose sec	
3	61	0	0				210
4	28	0	0	0			120
5	30	0	0	85			90
6	40	0		15		30	90
7	44	0	0	0			90
10	37	0	0	40			210
16	52	0	0	*		210	210
17	65	0				370	300
32	50	0			0		120
33	30	0	0		0	60	90
34	52	0	0		0		90
35	43	0	0				90
36	53	0	0				240

\*40 sec of shifting pacemaker (S-A and ventricular).

The control dose in each case was 0.01 mg adrenalin per kilogram. Fifteen minutes were allowed for recovery between injections of higher doses. All dogs recovered from Tachycardia.

in the dogs while subject to tachycardia was 140 mm Hg, and the average after protection had developed was 136 mm Hg. A response of ventricular tachycardia following the injection of adrenalin was always accompanied by a rise in blood pressure. After protection developed the response to adrenalin was a rise in pressure for 4 of the dogs and either no change or a reversal for the other 5. From the responses of the first 4 animals it is concluded that cyclopropane exerts its adrenolytic action on the heart before it has this effect on the peripheral vascular system. In the remaining 5 dogs cyclopropane had produced an adrenolytic effect upon both the heart and the blood vessels.

Repeated injections of adrenalin in doses of the magnitude used in this study cause comparable blood pressure rises in normal animals.<sup>6</sup> That this failure to obtain cyclopropane-adrenalin tachycardia is not due to the repeated injections of adrenalin is shown in Table I. Nine dogs had complete protection from ventricular tachycardia on the second injection; while after the fifth injection dogs 1, 3 and 13 had irregularities comparable in duration to those obtained with the first injection.

Furthermore, that the adrenolytic action of cyclopropane is not the result of general debilitation from prolonged anesthesia is evi-

<sup>6</sup> Sollmann, T., *A Manual of Pharmacology*, 5th ed., Saunders, 1940, p. 418.

TABLE III.  
Blood Pressure under Cyclopropane Anesthesia.

Dog No.	Minutes of cyclopropane	Blood pressure		Duration ventricular tachycardia sec
		Level before adrenalin (mm Hg)	Response to adrenalin (mm Hg)	
8	30	156	+ 34	45
	210	140	+ 30	0
10	30	154	+ 56	37
	210	142	+ 46	0
3	30	150	+ 80	18
	90	174	+ 58	0
17	30	100	+ 51	65
	300	140	+ 25	0
22	30	136	+ 74	70
	330	120	0	0
13	30	156	+104	71
	210	155	— 8	0
14	30	145	+ 25	29
	300	105	— 10	0
7	30	122	+ 38	48
	90	130	— 18	0
12	30	144	+ 76	70
	210	122	— 30	0

The dose of adrenalin in each injection was 0.01 mg per kilogram.

Direct blood pressure determinations were made from the femoral artery.

dent from the following: In Table I it is seen that 8 dogs developed protection with no more than 90 minutes of cyclopropane; while 9 dogs still gave a response after 5 hours anesthesia. Blood pressure levels before and after the development of protection are comparable as shown in Table III. An analysis of the electrocardiogram before the injection of the test dose of adrenalin did not indicate whether the adrenolytic effect had developed. There were no significant changes in the P and T waves and the QRS complexes. The average heart rate before the development of protection was 127 beats per minute as compared to an average of 153 after the development of protection.

*Conclusion.* Although cyclopropane initially sensitizes the dog's heart so that the injection of adrenalin causes ventricular tachycardia, subsequently it may exert an adrenolytic effect on the cardiovascular system.

## 13091

Separation of Thyrotropic and Interstitial Cell Stimulating  
(Luteinizing) Hormones of Ant. Pituitary.

H. JENSEN AND SIBYLLE TOLKSDORF.

*From the Biochemical Laboratory, The Squibb Institute for Medical Research,  
New Brunswick, N.J.*

*Introduction.* Preliminary chemical and physiological studies of the relationship between the thyrotropic and interstitial cell-stimulating (luteinizing) factors of the anterior pituitary indicated the possibility that these two principles are either identical or very closely related in their chemical aspects.<sup>1</sup> We would like to emphasize that we did not regard the evidence thus far obtained by us as proving definitely the identity of the two factors.

In the course of further chemical fractionation of our preparations we have obtained results which do not support the view of the identity of the two principles. We found that the interstitial cell-stimulating hormone can largely be freed from thyrotropic activity when precipitated repeatedly between 0.25 and 0.35 saturation of ammonium sulfate and that the thyrotropic potency is precipitated mainly between 0.35 and 0.5 salt saturation.

*Methods of Assay.* The interstitial cell-stimulating activity of the various fractions was determined in hypophysectomized immature female rats according to the method previously described.<sup>2</sup> The thyrotropic effect was assayed in 4-day-old chicks according to the procedure of Smelser<sup>3, 4</sup> as well as in hypophysectomized immature rats, and was determined by the histological stimulation of the thyroids rather than by the weight increase of that organ. It has been found that chick thyroids are more sensitive to thyrotropic hormone than the thyroids of hypophysectomized rats.<sup>5</sup> Comparison has been made of the minimal effective dose for interstitial cell stimulation in hypophysectomized rats and for stimulation of the thyroids in chicks.

*Experimental.* The starting material, obtained from sheep pituitary according to the procedure of Jensen, Simpson, Tolkstdorf and

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<sup>1</sup> Jensen, H., Tolkstdorf, Sibylle, *J. Biol. Chem.*, 1940, **133**, proceedings, p. 49.

<sup>2</sup> Jensen, H., Simpson, Miriam E., Tolkstdorf, Sibylle, and Evans, Herbert M., *Endocrinology*, 1939, **25**, 57.

<sup>3</sup> Smelser, G. K., *PROC. SOC. EXP. BIOL. AND MED.*, 1937, **37**, 388.

<sup>4</sup> Smelser, G. K., *Endocrinology*, 1938, **23**, 429.

<sup>5</sup> Frankel-Conrat, Jane, Frankel-Conrat, Heinz, Simpson, Miriam E., and Evans, Herbert M., *J. Biol. Chem.*, 1940, **135**, 199.



Evans,<sup>2</sup> was repeatedly precipitated with ammonium sulfate between 0.25 and 0.5 saturation and was found to have a comparatively high content of interstitial cell-stimulating and thyrotropic activity. Further fractionation with ammonium sulfate was carried out at a pH of 5 to 6 and a protein concentration of approximately 1%. An interstitial cell-stimulating fraction was obtained by repeated precipitation between 0.25 and 0.35 saturation as described by Jensen, Tolksdorf and Bamman.<sup>6</sup> This preparation was found to contain between 100 and 200 interstitial cell-stimulating units per mg and a thyrotropic potency up to 10%.\*

From the supernatants of the precipitate obtained between 0.25 and 0.35 ammonium sulfate saturation a fraction was isolated by repeated precipitation between 0.35 and 0.5 ammonium sulfate saturation which contained approximately equal amounts of interstitial cell-stimulating and thyrotropic potency. Traces of follicle-stimulating hormone were also found to be present. The minimal effective doses for interstitial cell-stimulating and thyrotropic potency found for representative samples of both fractions are illustrated in Table I.

*Discussion.* The experiments reported indicate the separate existence of an interstitial cell-stimulating and a thyrotropic hormone in sheep pituitary as demonstrated by their slightly different solubility in the presence of ammonium sulfate. These findings are in agreement with the observations recently reported from other laboratories. Chow, Greep and van Dyke<sup>7</sup> found that crystalline pepsin destroyed the thyrotropic hormone without seriously inactivating the interstitial cell-stimulating hormone. Fevold, Lee, Hisaw and Cohn<sup>8</sup> reported that the luteinizing (interstitial cell-stimulating)

TABLE I.  
Minimal Effective Doses for Interstitial Cell-Stimulating and Thyrotropic Potency.

Preparation	Hypophysectomized rats		Chicks
	Ovarian IT*	Thyroids	Thyroids
.25-.35 SAS†	10 γ	800 γ	160 γ
.35-.5 SAS†	50 γ	200 γ	40 γ

\*IT—interstitial tissue.

†SAS—saturation of ammonium sulfate.

<sup>6</sup> Jensen, H., Tolksdorf, Sibyle, and Bamman, Frieda, *J. Biol. Chem.*, 1940, **135**, 791.

\* A purified interstitial cell-stimulating hormone preparation kindly supplied by Dr. H. M. Evans gave similar results.

<sup>7</sup> Chow, B. F., Greep, R. O., and van Dyke, H. B., *J. Endocrinology*, 1939, **1**, 440.

<sup>8</sup> Fevold, H. L., Lee, Milton, Hisaw, F. L., and Cohn, E. J., *Endocrinology*, 1940, **26**, 999.

hormone is largely precipitated from sheep pituitary extracts at 2.0 M concentration of ammonium sulfate and the thyrotropic hormone at 2.4 M. Evans and his collaborators<sup>5</sup> have been able to obtain a thyrotropic fraction from anterior beef pituitary<sup>†</sup> by ammonium sulfate precipitation between 0.3 and 0.5 saturation. Further purification yielded a thyrotropic preparation containing 10% of interstitial cell-stimulating hormone.

*Summary.* The view of the identity of the interstitial cell-stimulating and thyrotropic pituitary factors could not be substantiated by the results obtained on further chemical fractionation of such preparations.

### 13092 P

#### Spontaneous and Reflex Emotional Responses Differentiated by Lesions in the Diencephalon.

MORRIS M. KESSLER.\* (Introduced by Claude S. Beck.)

*From the Department of Surgical Research, Western Reserve University.*

The diencephalic areas have been invoked as important subcortical centers having to do with the emotions.<sup>1, 2, 3</sup> The emotional reactions of animals with hypothalamic lesions have not been differentiated as spontaneous and reflex phenomena. The former appear without provocation and vary somewhat between different subjects. The reflex emotions occur only after stimulation of one or another sensory modality. The monkey is an excellent animal for study of spontaneous emotionality. He threatens, grimaces, wiggles his ears, undergoes pilo-erection, and vocalizes either in anger or in fear. When approached he may attempt to run away or permits his anger to carry him into definite aggressions. Occasionally, in blind rage, he dashes himself against the mesh of the cage as if he meant to rend it apart. Cats show a good deal less of spontaneous emotional display than do monkeys.

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† It is generally accepted that anterior beef and hog pituitaries contain more thyrotropic hormone than sheep pituitary.

\* Dazian Research Fellow.

<sup>1</sup> Grinker, Roy R., *Psychosomatic Med.*, 1939, **1**, 19.

<sup>2</sup> Ingram, W. R., *Psychosomatic Med.*, 1939, **1**, 48.

<sup>3</sup> Kabat, H., Anson, B. H., Magoun, H. W., and Ranson, S. W., *Am. J. Physiol.*, 1935, **112**, 214.

*Material and Method.* Monkeys and cats were used in this study. In all instances, the diencephalic lesions were placed under more or less direct vision by following the stump of one optic nerve to the chiasmal region.

*Observations.* 1. Total destruction of the hypothalamus.

Such a lesion invariably results in stupor in which there are no spontaneous emotional demonstrations. The stupor is frequently associated with cataleptic phenomena. When the animal is painfully stimulated, there is a prompt reflex display of emotions. The threshold to such reactions is elevated but the responses are certain. One cat responded to pressure over bony prominences, with baring of claws, opening of the jaws, an awesome rumbling in his throat, and a battery of sneezes. A similar preparation in a monkey could be aroused by stimulation to adequate shows of emotions including grimacing and defense movements of all extremities.

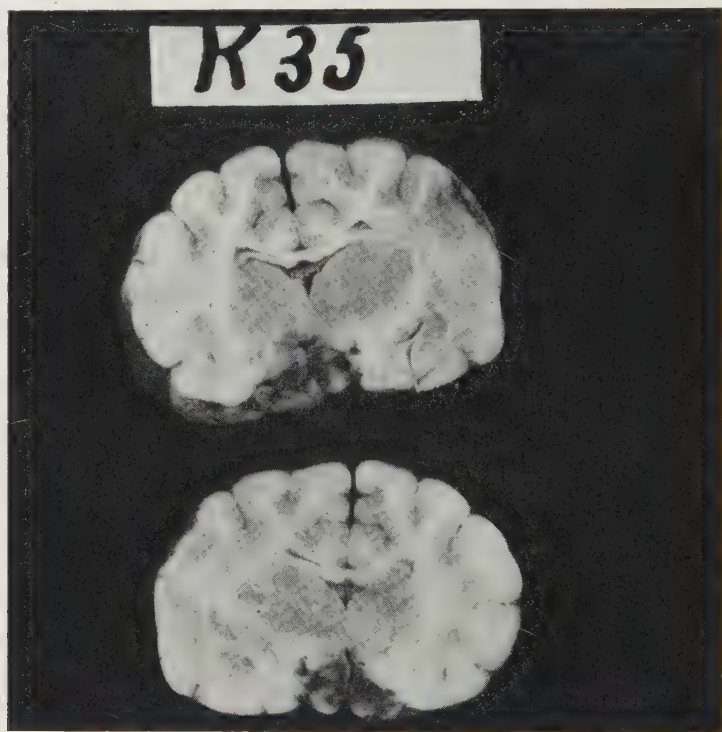


FIG. 1.

Total destruction of the hypothalamus in a cat. Microscopically, the lesion extends into the ventro-median area of the thalamus.

2. Total destruction of the hypothalamus along with involvement of the ventro-median portion of the thalamus.

Cats, so prepared, respond to painful stimulation hardly at all. One animal (Fig. 1), with only slight extension of the lesion into the ventro-median thalamus, responded with mere retraction of the lower lip and increase in the depth of respiration. A monkey could be roused to only slight defense movements of the limbs.

3. Unilateral destruction of the hypothalamus. This procedure results in an over-reactive animal who is conscious and ambulatory. There is a tendency to circle to the side of the lesion. One cat was constantly caterwauling. When baffled by anything in his way, he responded by baring his claws, snapping and spitting.

4. Unilateral destruction of the ventro-median portion of the thalamus. This procedure resulted in a spontaneously irritable and aggressively dangerous tabby which snapped and snarled and made wild swipes with bared claws at the examiner. This animal also was ambulatory and tended to circle to the side of the lesions.

*Discussion.* From these observations it would seem that where the hypothalamus is not functioning, there can be no spontaneous emotional display and stupor supervenes. This has been suggested previously by Ranson.<sup>4</sup> In such preparations, however, the thalamus is intact and is able to mediate pain stimuli along customary reflex arcs. This analogy may be carried over to stuporous human beings. The latter may be roused by painful stimulation to demonstrations of affect without actually regaining consciousness. One might postulate that in mild stupor the hypothalamus is not functioning, whereas the thalamus is physiologically intact. As the stupor deepens into coma, thalamic function is impaired and reflex emotional reactions may be no longer elicited. Anatomic correlates with this study indicate that the ventro-median nuclei of the thalamus play an important rôle in the elaboration of reflex emotional responses. Partial lesions of the hypothalamus or the ventral portion of the thalamus seem to produce an unusual lability of the subject's emotional display.

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<sup>4</sup> Ranson, S. W., *Harvey Lectures*, 1936-37, p. 92.



**Fibrinolytic Action of Gas Gangrene Anaerobes.\***

G. B. REED, J. H. ORR AND DOROTHY SMITH.

*From Queen's University, Kingston, Canada.*

Tillett's<sup>1</sup> review of fibrinolysis indicates the importance of the reaction in hemolytic *Streptococcus* infections. It also makes it apparent that various other species of bacteria produce enzymes or toxins which, though lacking some of the characteristics of *Streptococcus* fibrinolytic substance, tend to break down coagulated plasma and therefore influence the course of infection to a significant degree.

The very rapid spread of gas gangrene infections in man and experimental animals suggests that fibrinolysis may be involved. Accordingly a series of preliminary trials has been carried out with the more important species associated with gas gangrene in man. This included some 77 cultures belonging to eleven species, as listed in Table I.

*Cultures.* The cultures used were the same as those recently described by Reed and Orr.<sup>2</sup> Purity of the cultures was established by repeated plating shortly before use. All gave characteristic reactions according to the identification procedure as described in the last-mentioned paper. A few cultures were recently isolated from gas gangrene in man, a few were isolated from experimental gas gangrene in guinea pigs, Reed and Orr,<sup>3</sup> the majority were laboratory cultures which had not been through animal passage for long periods.

*Fibrinolytic Procedure.* The fibrinolytic test procedure was that used by Tillett and Garner<sup>4</sup> in the case of *Streptococcus*. Fresh oxalated plasma was obtained from man, rabbit, guinea pig and sheep. One milliliter of 1:5 dilution of oxalated plasma was mixed with 0.5 ml of culture, 0.35 ml of 0.25% calcium chloride added (0.25 ml for human plasma) the tubes well shaken and placed in a 37° water bath. Fibrinolysis readings were made at half-hour intervals, after coagulation, for the first 7 hours and again after 24 hours.

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\* Aided financially by a grant from the Canadian National Research Council.

<sup>1</sup> Tillett, W. S., *Bact. Rev.*, 1938, **2**, 161.

<sup>2</sup> Reed, G. B., and Orr, J. H., *War Medicine*, 1941, in press.

<sup>3</sup> Reed, G. B., and Orr, J. H., *Lancet*, 1941, March 22, 376.

<sup>4</sup> Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.

TABLE I.  
Fibrinolytic Activity of Gas Gangrene Bacteria.

Species	No. of cultures tested	Plasma	% of cultures producing solution of clot in	
			7 hr or less	24 hr or less
<i>Cl. welchii</i>	26	human	17	83
	33	rabbit	62	67
	5	guinea pig	80	100
	5	sheep	0	0
<i>Cl. novyi</i>	3	human	0	66
	3	rabbit	67	67
	3	guinea pig	0	67
	3	sheep	33	33
<i>Cl. septicum</i>	5	human	0	100
	5	rabbit	0	100
	3	guinea pig	100	100
	3	sheep	0	100
<i>Cl. sordellii</i>	4	human	0	80
	4	rabbit	0	0
	3	guinea pig	0	100
	3	sheep	0	0
<i>Cl. fallax</i>	1	human	0	0
	3	rabbit	0	0
	2	guinea pig	0	0
	2	sheep	0	0
<i>Cl. tertium</i>	1	human	0	0
	5	rabbit	0	0
	2	guinea pig	0	0
	2	sheep	0	0
<i>Cl. chauvoei</i>	2	human	0	50
	6	rabbit	0	17
	3	guinea pig	33	33
	3	sheep	0	33
<i>Cl. aerofaecium</i>	1	rabbit	0	0
	1	guinea pig	0	0
	1	sheep	0	0
<i>Cl. histolyticum</i>	4	human	25	100
	7	rabbit	86	86
	4	guinea pig	75	100
	4	sheep	25	50
<i>Cl. sporogenes</i>	4	human	0	100
	8	rabbit	63	88
	3	guinea pig	0	100
	3	sheep	0	33
<i>Cl. tyrosinogenes</i>	1	human	100	100
	2	rabbit	0	100
	1	guinea pig	100	100
	1	sheep	100	100

Several media provided suitable cultures. Brewer's medium with 0.1% glucose was first used but it was found that continuous cultivation in this medium resulted in a gradual decrease in fibrinolytic power. Most consistent results were obtained with the supernatant fluid from 18-hour cultures in chopped meat medium prepared according to the method of Lepper and Martin.<sup>5</sup> This medium was used in all cases reported in the paper.

*Fibrinolysis Results.* Table I summarizes the results with the several species in plasma from man, rabbit, guinea pig and sheep. The figures in the table indicate the percentage of cultures tested which break down coagulated plasma in 7 hours or less and in 24 hours or less.

It is apparent from the table that the 4 species principally concerned in gas gangrene, *i. e.*, *Cl. welchii*, *Cl. novyi*, *Cl. septicum* and *Cl. sordellii* produce active fibrinolysis. Guinea pig and rabbit plasma clots are slightly more readily broken down than human plasma while sheep plasma clots resisted action of *Cl. welchii* and sheep and rabbit plasma resisted *Cl. sordellii*. More than half the cultures of *Cl. chauvoei* were inactive on all 4 plasma.

It should be noted that cultures which break down plasma clots in 24 hours are regarded as fibrinolytically active. A considerable number produce complete solution of the clots, as shown in the table, in 7 hours or less. A few produce complete solution in one to 1½ hours. The most active were cultures of *Cl. welchii* recently isolated from acute cases of gas gangrene in man.

Three species occasionally found in gas gangrene wounds, *Cl. fallax*, *Cl. tertium*, *Cl. aerofœtidum* were completely inactive in all 4 plasma. These species produce little or no soluble toxin.

Three proteolytic species, *Cl. histolyticum*, *Cl. sporogenes* and *Cl. tyrosinogenes* were very active in the break-down of all 4 plasma. The former produces a low yield of toxin, the latter 2 no toxin.

The extent to which the fibrinolytic substances produced by these anaerobic species resemble the *Streptococcus* enzyme remains to be determined. It is quite possible, moreover, that the activity of the saccharolytic, toxin-forming species results from one type of enzyme and that the proteolytic, non-toxin-forming species break down plasma clots through the activity of a very different system. Regardless, however, of the resemblance of this digestive mechanism to that present in other species or the possible dissimilarity of mechanism in different groups of anaerobes, it is apparent that the gas gangrene species do rapidly destroy coagulated plasma. It is therefore

<sup>5</sup> Lepper, E., and Martin, C. J., *Brit. J. Exp. Path.*, 1924, **10**, 327.

highly probable that these reactions contribute to the spread of gas gangrene infections.

*Anti-coagulation Factor.* A few of the cultures from each fibrinolytic species regularly prevented clotting of plasma on the addition of  $\text{CaCl}_2$ ; and a few additional cultures occasionally prevented clotting. This condition was observed with a few cultures of *Cl. welchii*, *Cl. novyi*, *Cl. septicum* and even with a few of the proteolytic species—*Cl. histolyticum* and *Cl. sporogenes*. A similar anti-coagulating factor has been observed by Dennis and Adham,<sup>6</sup> Tillett,<sup>7</sup> Christensen,<sup>8</sup> and others in certain cultures of hemolytic *Streptococcus*. Tillett<sup>7</sup> considers this to result primarily from pH changes. Where the reaction is more acid than pH 5.8, plasma coagulation is inhibited. In these 18-hour cultures of anaerobes in chopped meat the reaction never exceeds pH 6.0 and generally is from pH 6.2 to 6.4. Dennis and Adham,<sup>6</sup> on the other hand, consider that the anti-coagulation factor in *Streptococcus* cultures is a particular fatty acid which may be independent of pH.

*Conclusion.* 1. It is shown that most strains of the more important saccharolytic, toxin-forming gas gangrene species, *Cl. welchii*, *Cl. novyi*, *Cl. septicum* and *Cl. sordellii*, produce an active fibrinolytic substance. Certain other saccharolytic species, *Cl. tertium*, *Cl. fallax*, *Cl. aerofetidum* fail to produce this substance. 2. The more common proteolytic gas gangrene species, *Cl. histolyticum*, *Cl. sporogenes* and *Cl. tyrosinogenes* produce an equally or more rapid destruction of coagulated plasma. 3. Most cultures which are active against plasma from one species will break coagulated human, guinea pig and rabbit plasma. Sheep plasma is less readily attacked and not attacked by any strains of *Cl. welchii* tested. 4. A few strains in both groups of gas gangrene anaerobes produce anti-coagulation factors which prevent  $\text{CaCl}_2$  coagulation of plasma. It is shown that this is not a pH effect.

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<sup>6</sup> Dennis, C. W., and Adham, L. D., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 84.

<sup>7</sup> Tillett, W. S., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 77.

<sup>8</sup> Christensen, L. R., J. Inf. Dis., 1940, **66**, 278.



# Effect of Desoxycorticosterone\* on Pituitary and Lactogen Content.†

C. W. TURNER AND JOSEPH MEITES.

*From the Department of Dairy Husbandry, University of Missouri, Columbia.*

Several of the sex hormones capable of eliciting mammary growth can also increase the weight of the pituitary and augment its content of lactogenic hormone. At least 2 estrogens, estrone and estradiol benzoate, have been shown to possess these properties.<sup>1</sup> Testosterone propionate, another mammary growth-promoting substance, can augment the lactogen content of the pituitary but does not increase the size of the gland.<sup>2</sup> Androsterone, which does not stimulate mammary growth,<sup>3</sup> fails to influence either the size of the pituitary or its lactogen content.<sup>4</sup>

Van Heuversvyn, *et al.*,<sup>5</sup> have demonstrated that desoxycorticosterone acetate (DCA), a synthetic adrenal cortical hormone, can induce growth in the mammary glands of male mice. Recently this has been confirmed by Lewis and Turner (unpublished). It was of interest, therefore, to determine whether DCA, like estrone, estradiol benzoate and testosterone propionate, would also increase the size of the pituitary or raise its lactogen content.

Forty-four guinea pigs were placed in 5 groups of experimental and control animals. From 7 to 20 mg of DCA in sesame oil were administered subcutaneously over periods ranging from 10 to 20 days. To show the comparative effects of estrogen, an additional group of 8 male guinea pigs were injected subcutaneously with 3000 I.U. of estrone over a period of 15 days and run against 6 control animals. The pituitaries of each experimental and control group were macerated separately in a small agate mortar, taken up in a little distilled water, and assayed by the Reece-Turner pigeon method.<sup>1</sup>

\* The desoxycorticosterone acetate was kindly furnished by Dr. Erwin Schwenk of the Schering Corporation.

† Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 751.

<sup>1</sup> Reece, R. P., and Turner, C. W., *Mo. Agr. Exp. Sta. Bul.* 266, 1937.

<sup>2</sup> Reece, R. P., and Mixner, J. P., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **40**, 66.

<sup>3</sup> Nelson, W. O., and Gallagher, T. F., *Science*, 1936, **84**, 230.

<sup>4</sup> Reece, R. P., *PROC. SOC. EXP. BIOL. AND MED.*, 1941, **46**, 265.

<sup>5</sup> Van Heuversvyn, J. E., Folley, S. J., and Gardner, W. U., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 389.

The data (Table I) relative to the size of pituitary and lactogen content was treated by the analysis of variance as described by Snedecor.<sup>6</sup> A significant increase in weight was found in the pituitaries of the DCA treated animals. However, the difference between the lactogen content of the pituitaries of the experimental and DCA treated guinea pigs was found to be insignificant. It will be noted that in the estrone-treated guinea pigs there was at least a three-fold increase in the lactogen content of the pituitary. The mammary glands of the female guinea pigs receiving the lowest dosage of DCA, 7 mg, were examined and compared with the mammary glands of its control mates by Dr. A. A. Lewis of this laboratory, and found to show considerable lobule hyperplasia. The dosages of DCA employed did not produce any noticeable change in the adrenal weights of the experimental animals.

It is concluded that DCA acts similar to some of the sex hormones to the extent that it can stimulate the growth of the mammary glands and increase the weight of the pituitary, but is unlike these hormones in that it does not cause an augmentation of the lactogen content of the AP.

*Summary.* Three groups of female and 2 groups of male guinea

TABLE I.  
Effect of DCA on Pituitary and Lactogen Content.

Sex	Guinea pigs No.	Treatment	Length of treatment, days	Avg body wt, g	Avg adrenal wt, mg	Avg pituitary wt, mg	Avg pituitary wt/100 g body wt	Avg No. R.T.* units per pituitary	Avg No. R.T. units /100 g body wt
♀	5	Controls		417	229.8	9.88	2.13	6.24	1.25
♀	5	7 mg DCA	10	427	201.2	12.36	2.84	7.10	1.66
♀	5	Controls		405	207.9	11.46	2.80	6.20	1.53
♀	5	15 mg DCA	15	439	206.7	15.48	3.53	6.75	1.53
♀	2	Controls		403	204.2	10.52	2.64	6.00	1.48
♀	2	20 mg DCA	20	419	213.6	14.95	3.56	5.75	1.37
♂	6	Controls		336		10.05	2.98	3.03	.90
♂	8	3000 I.U. estrone	15	304		10.87	3.57	12.75	4.19
♂	5	Controls		340	164.9	9.56	2.74	3.12	.91
♂	5	15 mg DCA	15	344	170.0	12.36	3.60	3.64	1.05
♂	5	Controls		205	69.4	5.71	2.79	2.90	1.41
♂	5	10 mg DCA	10	184	88.0	6.44	3.51	2.40	1.30

\*Reece-Turner lactogen units. 22.2 R.T. units = 1 International Unit (Meites, J., Bergman, A. J., and Turner, C. W., *Endocrinology*, 1941, **28**, 707).

<sup>6</sup> Snedecor, G. W., *Statistical Methods*, 3rd Edition, 1940, p. 179. We are indebted to Mr. M. Koger for the statistical analysis of the data.

pigs were injected with 7 to 20 mg of desoxycorticosterone acetate to determine its effect on pituitary weight and on the lactogen content of the AP. A significant increase in pituitary weight was obtained, but there was no increase in the lactogen content of the AP.

## 13095

**Effect of an Ant. Hypophyseal Extract upon Serum Calcium and Phosphorus.**

FRED H. SNYDER AND WILBUR R. TWEEDY.

*From the Department of Biological Chemistry, Loyola University School of Medicine, Chicago, Ill.*

The elaboration of a parathyreotropic factor by the anterior hypophysis has been indicated by work from a number of sources. The literature in this connection has been reviewed recently by Friedgood and McLean.<sup>1</sup>

These workers have presented data establishing a statistically significant increase in the serum calcium of guinea pigs receiving daily injections of 1 cc of an alkaline pituitary extract\* over a period of 9 days. Friedgood<sup>2</sup> has also published data indicating an elevation in the serum calcium level of white rats under similar conditions. In 4 rats, each of which had received daily subcutaneous injections of 1 cc of the extract for a period of 6 days, values for serum calcium were found to be 12.8, 12.6, 12.5, and 11.7 mg %, respectively. Two control animals showed serum calcium levels of 10.7 mg %. Four other rats were given intraperitoneal injections of 1, 2, 3, and 4 cc of the extract, respectively, twice daily and sacrificed on the second day. Their blood calcium values were found to be 12.7, 12.3, 10.1, and 10.3 mg %, respectively. No control values were reported.

In view of the small number of rats which were used to test the reactivity of this animal to the extract, and the inadequacy of the control values, it appeared to us that the suitability of the rat for the assay of the so-called parathyreotropic factor should be further investigated.

Albino rats, varying in age from 99 to 281 days and in weight

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<sup>1</sup> Friedgood, H. B., and McLean, R., *Am. J. Physiol.*, 1937, **118**, 588.

\* Obtained from E. R. Squibb and Sons.

<sup>2</sup> Friedgood, H. B., *Endocrinol.*, 1936, **20**, 159.

from 159 to 352 g, were used in the first assay. With the exception of 3 litter mate females, all the animals were males. Only one female was used in the experimental series. The animals were raised on a commercially prepared diet known as Fox Chow; in some instances a supplement of meat scraps was supplied twice weekly. Fox Chow alone was fed for several days preceding and during the course of each experiment.

Each of 20 experimental animals received a daily subcutaneous injection of 1 cc of an alkaline (pH 8.25) extract of anterior pituitary glands<sup>†</sup> for a period of 6 or 7 days. On the day following the last injection the animals were placed under light ether anesthesia and blood was drawn by cardiac puncture. Calcium was determined on 1 or 2 cc portions of serum by the method of Tweedy and Koch,<sup>3</sup> and inorganic phosphorus was determined on 1 cc portions of the same serum sample by the method of Fiske and Subbarow.<sup>4</sup> Control values were obtained by simultaneously carrying out the above analyses on samples of serum from 18 untreated litter mates of the experimental animals. Inorganic phosphorus values were obtained for 19 treated animals and 15 controls.

A statistical evaluation of the data (Table I) reveals that the calcium levels in the blood of the treated animals were not significantly affected by multiple injections of the anterior pituitary extract under the experimental conditions described. Furthermore, the small difference between the mean phosphorus values, when considered in relation to the probable error of this difference (Table I), may be regarded as only slightly suggestive of an increase in serum inorganic phosphorus.

TABLE I.

Type of animal	Max. mg%	Min. mg%	Mean* mg%	M.D.†	P.E.M.D.‡
Serum Calcium.					
Treated	12.50	8.32	9.62 ± 0.14	0.01	0.18
Control	12.48	8.81	9.63 ± 0.12		
Serum Inorganic Phosphorus.					
Treated	6.95	5.26	5.89 ± 0.07	0.27	0.08
Control	5.97	5.10	5.62 ± 0.04		

\*Including probable error of the mean.

†Mean difference.

‡Probable error of the mean difference.

† Obtained from E. R. Squibb and Sons through the courtesy of Dr. J. A. Morrell.

<sup>3</sup> Tweedy, W. R., and Koch, F. C., *J. Lab. and Clin. Med.*, 1929, **14**, 1.

<sup>4</sup> Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.



A group of 14 younger rats (52-54 days old) received from one to 3 daily subcutaneous injections of 1 cc of the hormone preparation, and 8 of their litter mates were used as controls. Approximately half of each group of these animals were females. Serum calcium values in the treated animals ranged from 9.7 to 11.4 mg % with a mean value of 10.8, while serum calcium values in the control series ranged from 9 to 12.2 mg % with a mean value of 10.7. In no instance did the serum calcium of a treated animal show a significantly greater value than that found for its litter mate control. Serum inorganic phosphorus determinations were not made.

*Summary.* A slight increase in serum inorganic phosphorus without a significant increase in serum calcium after multiple injections of anterior pituitary extract in rats is regarded as insufficient proof of the presence of a parathyreotropic factor in the extract examined.

### 13096 P

#### Nuclear Changes in Rous Sarcoma Cells Cultivated *in vitro*.

E. TENENBAUM AND L. DOLJANSKI. (Introduced by L. Halberstaedter.)

*From the Department of Experimental Pathology, Cancer Laboratories, The Hebrew University, Jerusalem.*

Various authors (Borrel,<sup>1</sup> Roskin,<sup>2</sup> Lipschütz,<sup>3</sup> Fischer,<sup>4</sup> Zweibaum<sup>5</sup>) have indicated that the structure of Rous sarcoma cells shows certain peculiarities, which differentiate them from normal mesenchyme cells.

These indications are very fragmentary and there is little agreement between the various descriptions. The morphological features of the Rous sarcoma cell are still not sufficiently known.

The structural peculiarities of the Rous sarcoma cells appear with great clarity and they are easily detectable in pure culture of sarcoma cells. Our studies of cell strains, cultured without addition of normal tissue revealed that the sarcoma cells *in vitro* constantly show pro-

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<sup>1</sup> Borrel, A., *C. R. Soc. Biol.*, 1926, **94**, 500.

<sup>2</sup> Roskin, G., *Virchows Arch.*, 1926, **261**, 919.

<sup>3</sup> Lipschütz, B., *Z. Kregsforsch.*, 1929, **28**, 491.

<sup>4</sup> Fischer, A., *Gewebezüchtung*, Munich, 1930.

<sup>5</sup> Zweibaum, J., *Arch. exp. Zellforsch.*, 1933, **14**, 358.

found morphological alterations of their cytoplasm and nuclei, often associated with marked, indeed extraordinary hypertrophy of the entire cells.

In this note we report on the changes taking place in the nuclei of the sarcoma cells. The studies were made on cultures (preparations *in toto* and serial sections) fixed in *Carnoy* and stained with *Giemsa*.

The changes in the nucleus are very profound and may lead to definite disturbances of the entire nuclear structure. The nuclear ground substances, which is finely reticulated, or finely granular in normal spindle cells, and almost structureless in normal lymphoid cells, in sarcoma cells often appears as if precipitated. In numerous nuclei particulate masses become visible. The individual particles are more or less uniform in size and stand out distinctly on the almost colorless background. At first the particles are equally distributed throughout the nucleus; but later the granular material gathers in the central parts of the nuclei, leaving a wide or narrow zone, a clear halo, between the particulate aggregates and the nuclear membrane (Fig. 1). This zone appears empty, but in some preparations we can see that it is crossed by very fine threads running radially. The granular material appearing in the nucleus is usually

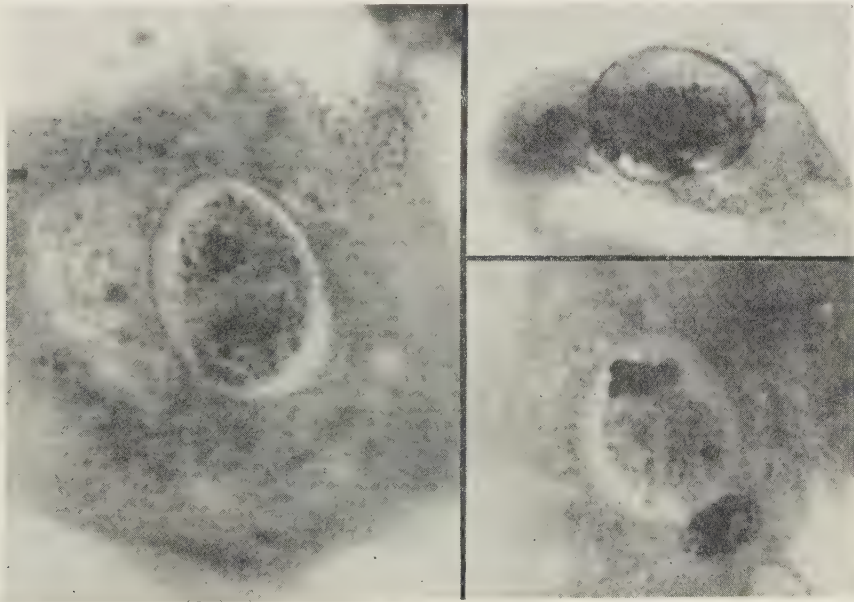


FIG. 1.  
Nuclei of Rous sarcoma cells; culture No. 12708; Giemsa's stain.  $\times 1600$ .

distinctly acidophilic. Occasionally the particulate masses are gathered not in the center but in certain peripheral areas of the nucleus. The remaining parts then appear empty.

The basophilic chromatin disappears progressively from the nucleus; the chromatin particles become smaller and less distinguishable. Margination of the basi-chromatin on the nuclear membrane is only rarely found.

The nucleoli usually become round or rod shaped, and appear as very large, compact, deeply stained, robust bodies. Often their shape is very irregular; not infrequently the nucleoli break up into several fragments; these either remain clumped together or may be found scattered throughout the entire nucleoplasm. Occasionally vacuoles are seen in altered nucleoli.

The changes described above do not take place simultaneously in all cells of a particular culture; in some the nuclei are profoundly changed; in others little or not at all. Apparently, the cells are involved successively.

In some cells, usually hypertrophied basophilic round cells, nuclear changes can be observed, different from those already described. Nuclei of such cells contain sharply defined inclusions, amorphous and hyaline in appearance, lying between the nuclear membrane and a nucleolus (Fig. 2). These inclusions are stained a bluish tint with Giemsa. The ground substance in which these inclusions are embedded is as a rule also altered.

In addition to the changes described the nuclei of sarcoma cells show a great tendency to fragmentation. The form of nucleus often

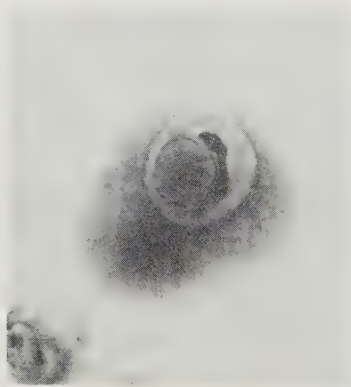


FIG. 2.

Intranuclear inclusion in Rous sarcoma cell; culture No. 12708; Giemsa's stain.  
X 660.

becomes irregular, parts are pinched off, and thus one can frequently find the nucleus broken up into 10-15 fragments; some are attached to each other by fine filaments. At times such fragments show a fully normal nuclear structure, others lack nucleoli, still others have no chromatin substance whatever, and some again appear completely emptied. Often the picture observed suggests that some nuclear fragments dissolve in the surrounding cytoplasm.

A detailed description of the changes in the nuclei of Rous sarcoma cells and a discussion of their significance will appear elsewhere.

### 13097 P

#### Cellular Composition of Pure Rous Sarcoma Cultures *in vitro*.

L. DOLJANSKI AND E. TENENBAUM. (Introduced by L. Halberstaedter.)

*From the Department of Experimental Pathology, Cancer Laboratories, The Hebrew University, Jerusalem.*

We wish to report on experiments undertaken in order to investigate the cellular composition of pure Rous sarcoma cell colonies *in vitro*. They were carried out on cultures, 20-50 passages old, of our Rous sarcoma strains, which were cultivated without the addition of normal tissue. They include observations on living cultures and on preparations *in toto* and serially sectioned ones, fixed in Carnoy or Zenker Formol, and stained with Giemsa.

The results of these investigations can be summarized as follows:

The pure Rous sarcoma culture consists of two cell types: spindle cells and round cells. The structure of the spindle cell corresponds exactly to that of the normal fibrocyte. The round cell is characterized by a strongly basophilic, completely homogeneous or finely granular cytoplasm and a relatively large, round, somewhat eccentric nucleus. The nucleus has a thick, unfolded membrane, 1-2 heavy nucleoli, and more or less finely divided chromatin. The size of the round cells varies between 5 and 15  $\mu$ . They are almost round, with regular or somewhat angular contours. Their pseudopodia are slightly developed, their movements are sluggish and they phagocytize but little.

The fibroblast-like cells and the basophilic round cells are not distinct cell types but different aspects of the same cell. In every culture numerous transitional stages enable us to follow, step by step,



the change of one cell form into the other. The transformation of the spindle cell into the basophilic round cell takes place by means of contraction of the former. The fibroblast-like cell draws in its processes; its body becomes round, the cytoplasm more and more basophilic, the nucleus becomes more compact, its membrane easily visible and the nucleoli very prominent. The basophilic round cell, arising from the contraction of the spindle-cells, recalls at times lymphocytes and even more haemocyto blasts as seen in cultures of blood and hemopoietic tissues. They differ distinctly from large, amoeboid polyblasts (macrophages) as well in form, nuclear structure, and cytoplasm as in their behavior. The process of transformation of spindle-cell into basophilic round cell is reversible; observations and experiments show that the basophilic cell can stretch itself out and recover the form of the spindle cell. Sometimes, the basophilic round cell may also take on an appearance closely resembling that of amoeboid histiocytes; it becomes progressively larger, its cytoplasm more and more vacuolated and pale; it acquires an amoeboid appearance and its pseudopodia become much better developed.

Our observations indicate a connection between the transformation of fibroblast-like cells into basophilic round cells and the peculiar fundamental ability of sarcoma cells to attack proteolytically and to liquefy their plasmatic growth medium. The change in the physical state of the medium surrounding the cell produces a change in its form. Whereas the cell is stretched out in a semi-solid medium, it becomes round when the plasma is liquefied. The sarcoma cell with its marked proteolytic activity itself creates the conditions that determine the diversity of its form. By adding or withholding embryonic extract, the proteolytic power of the sarcoma cell can be diminished or increased. In this manner the appearance of the sarcoma cells *in vitro* can, to a large extent, be experimentally influenced.

The fact that normal fibroblasts may undergo the same changes enables us to understand more fully the mechanism of transformation which the sarcomatous fibroblasts in culture undergo. Such changes in normal fibroblasts were first observed by Rous and Jones<sup>1</sup> when the plasmatic medium, in which the cells were grown, was digested with trypsin; and they can also be seen when an untreated culture of normal fibroblasts occasionally liquefied the medium spontaneously. Under these conditions the normal fibroblast too is transformed into

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<sup>1</sup> Rous, P., and Jones, F. S., *J. Exp. Med.*, 1916, **23**, 549.

a round cell, often practically indistinguishable from the basophilic round cell, resulting from transformation of the sarcomatous fibroblast.

In view of these findings, the discussion, which cell type in the sarcoma culture, the fibroblasts or the "macrophages" carries the malignant attributes, appears to be futile. The pure Rous sarcoma consisting of one cell type; a mesenchyme cell, appearing at times as a spindle cell, at others as a basophilic round cell is the actual carrier of the Rous sarcoma agent. The changes induced by the sarcoma agent in the spindle cells and basophilic round cells will be described in another communication.

## 13098

**Age Variations in Resistance of Albino Rat to Diphtheria  
Bacilli and to Diphtheria Toxin.**

ERICH SELIGMANN. (Introduced by C. W. Jungeblut.)

*From the Department of Bacteriology, College of Physicians and Surgeons,  
Columbia University, New York.*

As reviewed in a previous paper on experimental diphtheria in the albino rat<sup>1</sup> this animal possesses a high but not complete resistance to diphtheria toxin and to living diphtheria bacilli. Amounts of toxin exceeding by 2000 times the fatal dose for guinea pigs (calculated on body-weight) are required to kill albino rats by subcutaneous injection. Again, when large numbers of living bacilli are injected into these animals by the same route only slight transitory lesions are observed. Attempts to explain the mechanism of this natural resistance have been made by several authors. Thus Goodman,<sup>2</sup> Pettit,<sup>3</sup> Coca, Russel and Baughman,<sup>4</sup> Sbarsky<sup>5</sup> have thought that the rat's body-cells are incapable of fixing diphtheria toxin; Ledingham,<sup>6</sup> on the other hand, has expressed his belief that the reticulo-endothelial system of the rat is particularly capable of aiding in the absorption of localized abscesses in the subcutis

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<sup>1</sup> Seligmann, E., and Jungeblut, C. W., *J. Immunol.*, 1941, **40**, 119.

<sup>2</sup> Goodman, H. M., *J. Infect. Dis.*, 1907, **4**, 509.

<sup>3</sup> Pettit, A., *Ann. Inst. Pasteur*, 1914, **28**, 663.

<sup>4</sup> Coca, A. F., Russel, E. F., and Baughman, W. H., *J. Immunol.*, 1921, **6**, 387.

<sup>5</sup> Sbarsky, *Biochem. Z.*, 1926, **169**, 113.

<sup>6</sup> Ledingham, J. G. C., *J. State Med.*, 1926, **34**, 2.

and to prevent a dissemination of the bacilli. However, that at least a temporary generalization of the bacilli does occur was suggested by our experiments in which it proved possible to recover diphtheria bacilli from the spleen of infected animals.<sup>1</sup>

The assumed peculiarities of the body-cells in that respect may be connected either with some general phenomena of metabolism or with the specialized function of certain organs that are supposed to be involved in the mechanism of diphtheria intoxication. Since the adrenal glands, for a long time, have been known to be heavily affected in experimental diphtheria, attempts have been made to clarify the significance of this organ for the resistance of rats to toxin. It was found, for instance, that adrenalectomized rats succumbed to smaller doses of diphtheria toxin than controls which had been similarly operated on but without removal of the adrenals (Lewis,<sup>7</sup> Belding and Wyman<sup>8</sup>).

The lesions in the suprarenal glands in diphtheria intoxication of guinea pigs are known to be accompanied by a decrease of the vitamin-C contents of this organ (Harde,<sup>9</sup> Torrance<sup>10</sup>). That this vitamin may play a definite part in the interaction between toxin and tissue was indicated by the investigations of Jungeblut and Zwemer,<sup>11</sup> Greenwald and Harde,<sup>12</sup> and lately again by Jungeblut,<sup>13</sup> who demonstrated that neutral salts of ascorbic acid detoxified diphtheria toxin *in vitro*. It is generally recognized that C-avitaminosis cannot be produced experimentally in the albino rat because this animal synthesizes this vitamin by itself; however, a diet deficient in vitamin A or B produces a definite avitaminotic state. In experiments by Werkman, Baldwin and Nelson<sup>14</sup> such avitaminosis was accompanied by a marked decrease in resistance to diphtheria toxin. An increased resistance, on the other hand, was demonstrated by Meyer<sup>15</sup> in the course of a high-protein diet. Since a similar increase could be observed following inanition the latter was

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<sup>7</sup> Lewis, J. T., *Am. J. Physiol.*, 1923, **64**, 506.

<sup>8</sup> Belding, D. L., and Wyman, L. C., *Am. J. Physiol.*, 1926, **78**, 50.

<sup>9</sup> Harde, E., *C. R. Acad. Sciences*, 1934, **199**, 618.

<sup>10</sup> Torrance, J. *Biol. Chem.*, 1940, **32**, 575.

<sup>11</sup> Jungeblut, C. W., and Zwemer, R. L., *PROC. SOC. EXP. BIOL. AND MED.*, 1935, **32**, 1229.

<sup>12</sup> Greenwald, C. K., and Harde, E., *PROC. SOC. EXP. BIOL. AND MED.*, 1935, **32**, 1157.

<sup>13</sup> Jungeblut, C. W., *J. Infect. Dis.*, in press.

<sup>14</sup> Werkman, C. H., Baldwin, F. M., and Nelson, V. E., *J. Infect. Dis.*, 1924, **35**, 549.

<sup>15</sup> Meyer, A. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 404.

therefore regarded as operating essentially as equivalent to a high-protein diet.

It is generally acknowledged that diet and vitamin-storage are intimately connected with the rate of growth of young animals. Thus the suckling period represents a course of high-protein diet; at the same time the vitamin-C content of the adrenals has been found appreciably lower in young rats than in adult animals.<sup>16</sup> Moreover, the lack of differentiation in the cells of the spleen in very young rats<sup>17</sup> may be of additional significance for the mechanism of natural defense. It has, therefore, seemed justified to investigate the degree of natural resistance to diphtheria bacilli and to toxin in very young rats, all the more so since an increased susceptibility of young animals to diphtheria toxin has already been reported for guinea pigs, dogs, birds, and white mice.<sup>18</sup>

*Experiment 1.* Bacillary infection: Rats, 10 days of age (average weight 12 g), and rats just weaned, 25 days old, (average weight from 40 to 60 g) were infected by subcutaneous injection with 1/10 of the growth of a Loeffler slant (3.8" x 0.3" in size) emulsified in saline. When these rats were killed at intervals of 2, 4, 7, and 9 days after infection these animals presented lesions similar to those typical for adult rats. They consisted of a small encapsulated abscess at the site of the injection which contained pus as well as free and phagocytized diphtheria bacilli; at times cultivable diphtheria bacilli were found in the spleen. Rats that had not been killed survived in apparently good health. Thus no difference could be detected in the natural resistance of albino rats to bacillary infection whether the animals were of suckling age, just weaned, or had reached maturity.

*Experiment 2.* Intoxication: The effect of diphtheria toxin was studied in 3 groups of rats: 20 animals of suckling age (12 days old and weighing from 12 to 30 g), 12 rats which had just been weaned (25 days old and weighing from 33 to 48 g), and 15 adult rats (125 to 265 g in weight). All animals received varying doses of toxin by the subcutaneous route. The results are given in Table I.

The table reveals an increased susceptibility of young rats to diphtheric intoxication. Thus, suckling animals died with a minimal dose of 3 mld per g of body-weight, weaned animals of somewhat higher age and weight required about 5 mld, while adult

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<sup>16</sup> Clark, A. R., personal communication.

<sup>17</sup> Perla, D., in Perla, D., and Marmorstone, J., *Natural Resistance and Clinical Medicine*, Boston, Little, Brown & Co., 1941, p. 356.

<sup>18</sup> Ssacharoff, G. P., *Ergebn. d. allg. Pathol. und pathol. Anat.*, 1928, **22**, 201.



TABLE I.  
Susceptibility of Albino Rats of Different Age and Weight to Diphtheria Toxin.

Avg age	Wt, g	Toxin in mld per g	No. of animals	Survived	Succumbed	Avg incubation period, days
12 days	12-30	< 1	6	6	—	—
		1	2	2	—	—
		2	3	3	—	—
		3	3	—	3	5.7
		4	2	—	2	5.0
		5	1	—	1	4.0
		6	3	—	3	2.3
25 "	33-48	3	3	3	—	—
		4	3	2	1	7.0
		5	3	1	2	7.0
		6	3	—	3	8.3
Adult rats	125-265	5	2	2	—	—
		6	2	2	—	—
		7	3	1	2	8.0
		8	8	—	8	5.6

rats succumbed only to doses of at least 7 mld. The dead rats showed a hemorrhagic edema of the lungs, frequently pleural effusion and sometimes congestion of the adrenal glands.

These data indicate that there may be a difference in age-resistance to diphtheria toxin but not to bacillary infection. This fact is readily explained since even the most susceptible suckling animal succumbs only to comparatively large doses of toxin, *i. e.*, 3 mld per g body weight. It is not to be assumed that such an amount of toxin ever accumulates in the animal's body during the course of experimental infection with diphtheria bacilli.

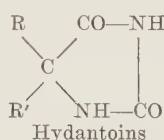
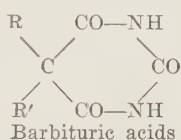
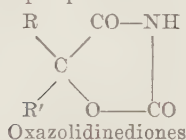
*Conclusion.* Young albino rats are more susceptible to diphtheria toxin than are adult animals. No difference seems to exist between young and old rats regarding their resistance to bacillary infection.

# Clinical Studies on the Hypnotic Properties of Propazone (5,5-Di-n-propyl-2,4-oxazolidinedione).

FRANK H. LUTON, JOHN BLALOCK, J. H. BAXTER, JR., AND ROGER W. STOUGHTON. (Introduced by P. D. Lamson.)

*From the Departments of Psychiatry and Pharmacology, Vanderbilt University School of Medicine, Nashville, Tenn.*

A series of new chemical compounds 5,5-dialkyl-2,4-oxazolidinediones have recently been shown by Stoughton<sup>1</sup> to have hypnotic and anesthetic properties. These are structurally related to the barbituric acids and to the hydantoins and have chemical and physical properties very similar to them.



Oxazolidinediones are mostly low-melting solids very slightly soluble in water, but on account of the acidic nature of the hydrogen on the nitrogen, they form stable water-soluble sodium and calcium salts, the solutions of which are stable to boiling. As yet, only 2 types of these oxazolidinediones have been studied pharmacologically. In the first, R is methyl, while R' consists of different alkyl radicals. In the second type, both R and R' are identical alkyl groups.

All of these substances, when given intravenously to animals, produce a certain degree of anesthesia which is usually of very short duration. Although the different members of these and other types of oxazolidinediones are being investigated further by Stoughton, the di-n-propyl-, to which we have given the tentative name "Propazone", differed so greatly in its effects on animals that it was chosen as the first to be studied more carefully in order to determine if it was suitable for clinical trial. A detailed report of the chemical properties of "Propazone" and its pharmacological actions can be found in the papers by Stoughton<sup>1</sup> and Stoughton and Baxter.<sup>2</sup> This may be briefly summarized as follows:

When dogs were given a sufficient amount of Propazone to produce a state of complete surgical anesthesia (125-175 mg per kg) the anesthesia lasted for about half an hour. During the next 15

<sup>1</sup> Stoughton, submitted for publication.

<sup>2</sup> Stoughton and Baxter, *J. Pharm. and Exp. Therap.*, 1940, **69**, 304.

to 20 hours, the animal remained in a state of deep hypnosis, from which it could not be completely aroused, although reacting to painful stimuli. This was followed by a period of lighter hypnosis, of about the same duration, during which time the animal remained asleep but could be aroused sufficiently to take food and water, although unable to stand and walk about. There was practically no effect on blood pressure or respiration if the drug was slowly injected. No change in the electrocardiogram was observed. Dogs were given intravenously 8 or 10 doses of Propazone, varying in size from 25-250 mg per kg, at intervals varying from 5 days to 2 weeks. Although these animals were completely anesthetized by most of these injections, they all recovered and showed no visible signs of injury or any pathological changes in the blood or urine. They were killed after various intervals and sections made of several tissues but no pathological changes were found.

The smoothness with which anesthesia could be produced, the very prolonged period of hypnosis which followed and the absence of side effects seemed to justify a clinical trial of this substance.

A group of psychiatric patients was chosen for study. All of them were in good physical condition and their behavior was such that modification by the drug could be readily recognized.

As there is practically no latent period before the onset of action of Propazone, the intravenous route of administration was chosen. In this way, it was possible to follow very exactly the effects of definite amounts of the drug and to stop its administration at any moment. A 10% solution of Propazone\* as the sodium salt was used in these cases. Amounts varying from 12 mg to 100 mg per kg of body weight were given intravenously at speeds varying from 1.2 to 3.3 cc per minute. In all, 22 intravenous injections were made in 11 patients. The blood pressure, pulse rate and respiratory rate were recorded during the injection and for some time afterwards. No significant change in any of these factors was observed when the drug was injected slowly. If it was injected too rapidly, there was a burning sensation in the arm and an increase in the pulse rate as well as a slight fall of blood pressure, but there was no apparent effect on the respiration. Such signs and symptoms passed off at once if the rate of injection was slowed. Two cubic centimeters per minute seemed to be the optimum injection rate as it caused no untoward effects in any case.

It should be pointed out that a definite fall in blood pressure may

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\* For the early experiments, this substance was prepared in our laboratory, but was later supplied to us through the kindness of the Mallinckrodt Chemical Works.

occur if the drug is injected too rapidly. This was observed when determining the optimum rate of injection. One patient in whom 44 mg per kg of Sodium Propazone was injected intravenously at a slow rate showed no untoward effects whatever. Ten cubic centimeters more of the 10% solution was injected rapidly and the systolic blood pressure fell from 110 to 80 mm of Hg. The blood pressure was immediately restored by the subcutaneous injection of nikethamide (coramine). This same effect is seen after too rapid injection of other hypnotics and should serve to emphasize the need of caution with regard to the rate of injection.

Drooping of the eyelids occurred with doses of about 5-10 mg per kg. After such amounts of the drug, the patients stated that they were not sleepy, but when left alone, were found to have slept more than normally. With doses of approximately 10 to 20 mg per kg, there was pronounced relaxation, although the patients were readily accessible. After such doses, a sleep of 2 to 5 hours followed. Loss of consciousness usually occurred after the injection of 30-50 mg per kg, but the patient could be aroused by mechanical stimuli, as pinching. If left alone, they slept for 8 to 10 hours. Complete anesthesia from which the patient could not be aroused by mechanical stimuli was obtained with doses of 50-100 mg per kg. These patients could be aroused after a few hours and were able to take food 8 or 10 hours later, but they immediately went back to sleep. One patient remained under the influence of the drug for 30 hours. A summary of the results obtained is given in Table I. No figures are given for the duration of anesthesia or hypnosis as it was impossible to observe all the patients continuously after treatment. (See table.)

These patients were followed for several weeks after treatment and showed no ill effects from the administration of Propazone. The urine was examined for sugar, albumin and casts, and showed no pathological changes.

A small group of epileptics who had been receiving luminal daily were given Sodium Propazone by mouth in doses of 1 g twice daily to replace the luminal. In all instances there was a reduction in number and in severity of attacks. One patient was given an intravenous injection during a convulsion. After about 5 cc of the 10% solution were given, she became completely relaxed and talked to the physician.

A patient with marked choreiform movements associated with episodes of crying and noisiness showed definite improvement in behavior when given the drug by mouth.



TABLE I.

Patient	Wt, kg	No. of inj.	Dosage, mg/kg	Rate of injection, cc/min	Degree of change during or immediately following injection		
					Blood pressure, mm of Hg	Pulse per min	Respiration per min
1	80	1	100	2.0	106/60-128/76	74-96	20-23
2	57	2	61	3.3	118/74-80/50	110-82	18-15
			37	1.2	120/70-106/70	87-96	20
3	64	1	34	1.1	120/80-117/76	78-88	18-17
4	47	1	81	1.8	88/56-108/70	75-86	22-18
5	57	2	100	2.1	90/58-108/72	90-110	20-18
			53	2.1	110/70-106/68	86-97	*
6	83	7	12	2.9	120/56-116/50	84-72	18-20
7	64	1	17	3.3	104/68-102/62	68-72	*
8	65	3	38	2.2	116/68-110/66	84-69	18-17
			19	1.9	112/70-108/60	82-80	*
			31	2.3	122/74-118/74	80-84	*
9	47	1	21	1.2	118/70-112/68	82-90	*
10	44	1	42	2.3	100/66-104/70	92	*
11	50	2	60	2.0	100/66-104/71	88-92	20-18
			40	—	—	—	—

\*Not taken.

*Conclusions.* Propazone (5,5-dipropyl-2,4-oxazolidinedione) has been given intravenously and by mouth to a group of patients. All stages of hypnosis and anesthesia have been produced without ill effects. The absence of side reactions, the striking degree of relaxation which comes on in even the early stages of hypnosis and its long duration of action indicate that this substance might be of therapeutic value. Its use in epilepsy is being investigated.

## Effect of Adrenal-Demedullation on Acceleration of Denervated Heart by Acetylcholine Hypotension.\*

W. B. YOUMANS, H. F. HANEY, A. J. LINDGREN AND A. I. KARSTENS.

*From the Department of Physiology, University of Oregon Medical School, Portland.*

Evidence has been presented that adrenaline and sympathin are liberated reflexly in unanesthetized dogs as a result of the brief hypotension produced by intravenous injection of acetylcholine.<sup>1</sup> The diphasic action of acetylcholine on intestinal motility was interpreted as resulting from a direct stimulatory effect of the compound on the intestinal smooth muscle followed by inhibition of the intestine by sympathomimetic substances. Adrenal demedullation prolonged the direct excitatory phase and reduced the inhibitory phase. It was considered that the inhibition that still resulted after adrenal demedullation was caused by sympathin liberated at the endings of adrenergic nerves activated reflexly by the fall in blood pressure.

Wiggers and Green have suggested that the evidence that a part of the acceleration of the denervated heart after acetylcholine injection is caused by adrenalin would have been more convincing had it been shown that the acceleration is reduced after excision of the adrenal medullae.<sup>2</sup> Such an experiment should also determine the cardiac effects of the sympathin produced during acetylcholine hypotension. The effect of a given dose of acetylcholine on the rate of the denervated heart can not be readily determined for the same dog before and after adrenal-demedullation, because the nutritional state of the animals with denervated hearts does not permit carrying them through additional operations. However, records have been obtained from 2 series of dogs with denervated hearts, one series having intact adrenal glands and the other series having the adrenals demedullated prior to the cardiac denervation.

Data obtained by the methods previously described<sup>1</sup> show that adrenal demedullation greatly reduces, but does not entirely eliminate, the acceleration of the denervated heart following acetylcholine in-

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\* Aided by a grant from the John and Mary R. Markle Foundation.

<sup>1</sup> Youmans, W. B., Aumann, K. W., Haney, H. F., and Wynia, F., *Am. J. Physiol.*, 1940, **128**, 467.

<sup>2</sup> Wiggers, C. J., and Green, H. D., *Annual Rev. Physiol.*, 1941, **3**, 313.

jections. These facts provide additional evidence that acetylcholine causes the liberation of sympathomimetic substances from both adrenal and extra-adrenal sources. The lower curves in the figure show a comparison of the average effect of intravenous acetylcholine on the rates of the denervated hearts of otherwise normal dogs (solid line) and of dogs with the adrenal glands demedullated (broken line). The former curve is based on 28 experiments on

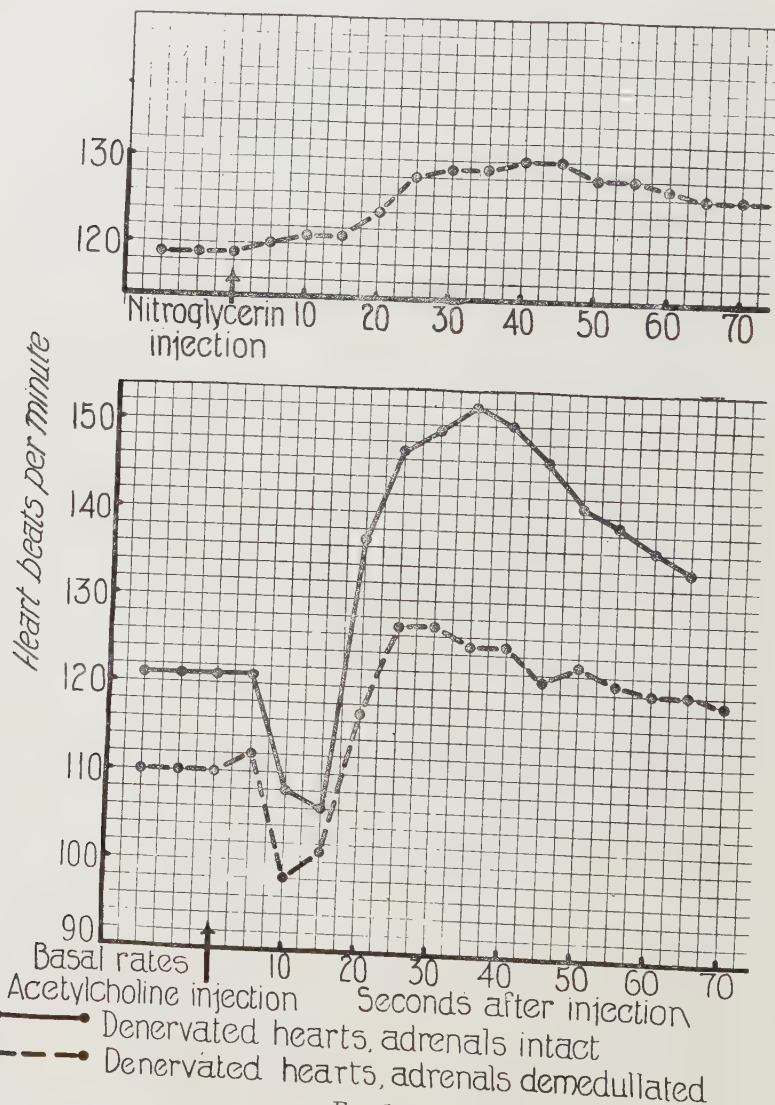


FIG. 1.

9 dogs, and the latter is based on 15 experiments on 5 dogs. Analysis of individual records showed that the denervated hearts of adrenal-demedullated animals were accelerated to rates 10% to 35% above the pre-injection level. The denervated hearts of animals with adrenals intact were accelerated, with comparable doses, to as high as 100% above the pre-injection level, and half of these were accelerated more than 35%. Hearts were considered to be completely denervated if they showed no reflex slowing from a rise in blood pressure produced by neosynephrin and if early acceleration was not obtained following a sharp fall in blood pressure. Eight dogs were discarded because they failed to meet these requirements.

It is possible that a part of the sympathin production during acetylcholine hypotension may have resulted from ganglionic stimulation. Therefore, it was desirable to produce hypotension by a drug lacking nicotinic action. A dose of nitroglycerin was determined which upon intravenous injection produced a severe lowering of blood pressure. The effect of this dosage (1 cc of 1 to 2500 solution) on the rate of the denervated heart as determined in 3 adrenal-demedullated dogs is shown in the upper part of the figure. The late appearance of the cardiac acceleration and its duration indicate that the acceleration is not caused by the direct action of the nitroglycerin. The animals showed no external signs of being disturbed by the injection.

The data obtained in this study when combined with those of the previous paper<sup>1</sup> indicate that a sharp lowering of the blood pressure produced by mild procedures in unanesthetized dogs under near-basal conditions is opposed by reflex liberation of adrenalin. When the adrenals are demedullated a lesser amount of a substance acting qualitatively and quantitatively like adrenalin on both the denervated heart and the denervated intestine still enters circulation. This substance is probably sympathin produced primarily by excitatory adrenergic nerves which are reflexly activated to compensate for the low blood pressure. However, this sympathomimetic substance does not possess the peculiar properties attributed to excitatory sympathin by the theory of Cannon and Rosenblueth;<sup>3</sup> it can not be distinguished from adrenalin itself by the test objects used.

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<sup>3</sup> Cannon, W. B., and Rosenblueth, A., *Autonomic Neuro-effector Systems*, pp. 98-109, Macmillan & Co., 1937.



## 13101 P

**Excretion of Bromsulfalein in the Bile.**

A. CANTAROW AND C. W. WIRTS.\*

*From the Departments of Medicine and Physiology, Jefferson Medical College, and the Laboratory of Biochemistry, Jefferson Hospital, Philadelphia.*

Bromsulfalein, injected intravenously in a dosage of 2 mg per kilo of body weight, is rapidly removed from the blood stream (85-95% in 5 minutes; 100% in 30 minutes), but its elimination in the bile continues over a period of several hours. This suggests that two separate or related mechanisms are involved and that simultaneous investigation of the rapidity of removal of the dye from the blood and the curve of its elimination in the bile might yield information of interest.

The concentration of bromsulfalein in the bile was determined as follows: 0.1 cc of bile was added to 15 cc of distilled water and 0.1 cc of 10% NaOH, and mixed by inversion. A blank was prepared for each specimen, containing 0.1 cc of bile, 15 cc of water and 0.1 cc of 10% NaCl. If turbidity developed the mixtures were filtered. After 10 minutes, readings were made in the Evelyn photoelectric colorimeter, using filter 580, and the concentration of bromsulfalein determined on the basis of a calibration curve obtained by adding known amounts of the dye to bile. In the case of very high concentrations, 30-45 cc water may be used instead of 15 cc.

Studies were made upon 6 cholecystectomized, bile-fistula dogs (45 determinations), 10 patients with T-tubes in the common bile duct (32 determinations), and 24 subjects in whom bile was obtained by duodenal intubation (60 determinations). Bromsulfalein was injected intravenously (2 mg per kilo), blood was withdrawn at the end of 30 minutes for estimation of the degree of retention of dye and determination of the serum bilirubin concentration, and bile was collected by continuous drainage in 15-minute fractions over a period of at least 2 hours. In patients in whom the gall bladder was present, the bladder bile was withdrawn (magnesium sulfate stimulation) before injection of the dye.

Under normal conditions, none of the dye remained in the blood at the end of 30 minutes after injection, and it usually appeared in the bile during the first 15-minute collection period. A maximum concentration in the bile (24-200 mg per 100 cc in human subjects;

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\* Ross V. Patterson Fellow in Gastroenterology.

42-292 mg per 100 cc in dogs) was reached in 45-75 minutes, the quantity of dye subsequently falling to a relatively low level at 2 hours, some frequently being still present after 5-6 hours. Normally, 50-83% of the quantity injected was excreted in the bile during the first hour and 67-100% within 2 hours. The highest concentration observed in the entire series (normal and abnormal) in bile obtained directly from the common duct was 532 mg per 100 cc in dogs and 369 mg per 100 cc in human subjects, while the highest concentration in material obtained by duodenal intubation was 200 mg per 100 cc. The frequently lower concentrations in the latter cases in the presence of normal total excretion values is probably due to admixture of the bile with pancreatic, duodenal and, occasionally, gastric secretions. Contamination with gastric juice, which may cause considerable turbidity in the specimen, may be minimized by the use of a double lumen tube (Diamond),<sup>1</sup> permitting continuous removal of gastric secretion under negative pressure through one channel simultaneously with withdrawal of duodenal contents through the other.

Abnormal excretion of the dye was evidenced by one or more of the following phenomena: (1) delayed removal from the blood; (2) delayed entrance into the bile; (3) delayed attainment of maximum concentration in the bile; (4) prolonged high curve of excretion in the bile; (5) subnormal concentration in the bile; (6) abnormally low excretion within one or 2 hour periods following the injection.

In 9 patients with other evidence of hepatic or biliary tract disease, significantly abnormal findings were obtained in the absence of abnormal retention of bromsulfalein in the blood. In these cases, the one-hour excretion ranged from 6 to 45% and the 2-hour excretion from 30 to 75% of the amount administered. Similar findings were obtained on 11 occasions in bile-fistula dogs, the total excretion at one hour being 25.7-41.1% and at 2 hours, 36-61% of the quantity administered.

Observations were made during a period of progressive partial obstruction to the flow of bile in a bile-fistula dog. The latter findings are presented in Table I.

These data indicate that considerable flattening of the curve of dye excretion in the bile, and progressive diminution in the total quantity excreted during the 2-hour period may occur before there

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<sup>1</sup> Diamond, J. S., Siegel, S. A., Gall, M. B., and Karlen, S., *Am. J. Digest. Dis.*, 1939, **6**, 355.

TABLE I.  
Bromsulfalein Excretion in a Bile-Fistula Dog During Progressive Partial Biliary Obstruction.

Date	Blood		Minutes								Total Excretion	
	Bili-rubin mg %	Dye %*	15	30	45	60	75	90	105	120	1 hr	2 hr
			Mg dye per 100 cc bile								%	%
2-4	.1	0	29	292	210	100	97	95	95	77	83	91
2-6	.1	0	1	59	159	150	127	111	76	55	35	61
2-10	.3	0	2	19	46	45	45	33	25	30	26	36
2-14	.9	0	0	3	4	19	18	33	35	38	4	18

\*30 minutes after injection of 2 mg per kilo.

is any significant interference with removal of the dye from the blood.

There is some evidence that Kupffer and other reticuloendothelial cells are concerned with the removal of bromsulfalein from the blood,<sup>2-5</sup> whereas its elimination in the bile must be accomplished by the hepatic cells. The procedure presented here affords a means of clinical investigation of these two phases of the elimination of bromsulfalein from the body. The data suggest that dissociation of Kupffer and hepatic cell function may be demonstrated frequently, particularly during periods of increasing and decreasing bile stasis. Other studies are in progress which are concerned with the effects of reticuloendothelial "blockade" and the administration of various cholagogic and choleretic agents.

### 13102

#### Some Effects of Injection of Acacia: With Special Reference to Renal Function.

ARNOLDUS GOUDSMIT, JR., MARSHELLE H. POWER AND JESSE L. BOLLMAN.

*From the Divisions of Biochemistry and Experimental Medicine, Mayo Foundation, Rochester, Minnesota.*

In recent years numerous reports have appeared in the literature relative to the diuretic activity of acacia in certain forms of renal disease associated with edema. Similarly, many experimental studies

<sup>2</sup> Herlitz, C., *Acta, paediat.* (supp. 5), 1931, **12**, 1.

<sup>3</sup> Klein, R., and Levinson, S. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1933, **31**, 179.

<sup>4</sup> Mills, M. A., and Dragstedt, C. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1936, **34**, 228.

<sup>5</sup> Mills, M. A., and Dragstedt, C. A., *Arch. Int. Med.*, 1938, **62**, 216.

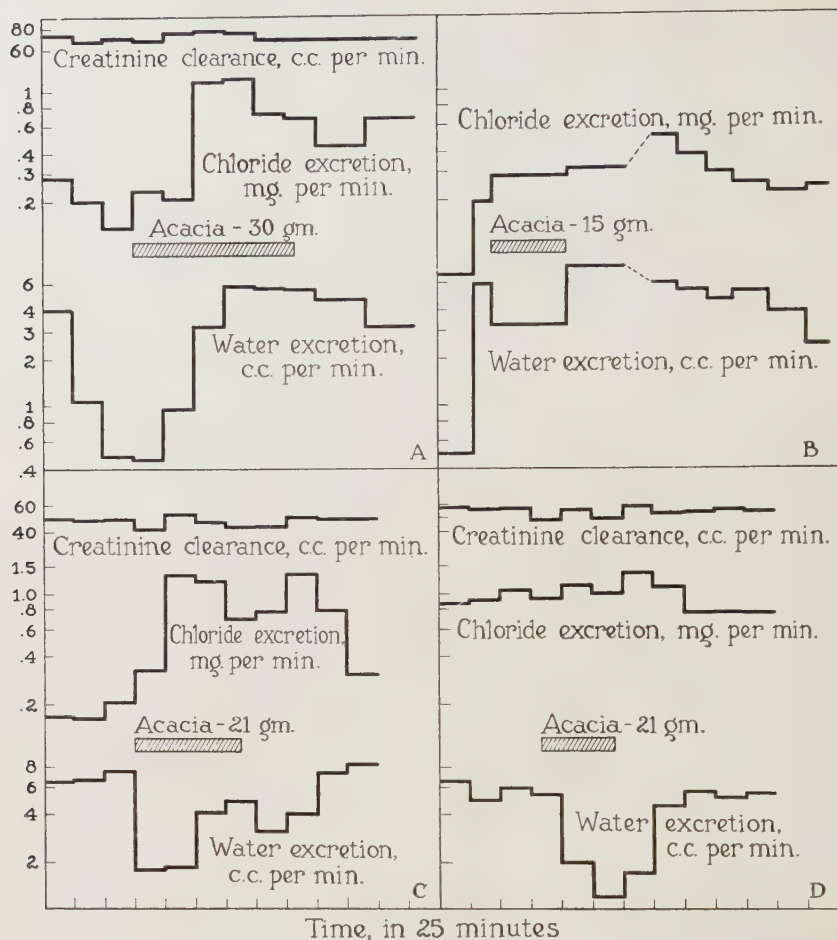
have been published dealing with the effect of the administration of solutions of acacia on the volume of the blood and plasma, the concentration of plasma proteins and the function of the liver. No report of its action on the functions of the kidney, however, has come to our attention. The present experiments were undertaken to obtain such information.

*Experimental.* A 5% solution of d-glucose was injected intravenously into dogs at a constant rate throughout the experiments. After steady diuresis had been established, an autoclaved salt-free 10% solution of acacia in 5% d-glucose was infused instead, without changing the rate of injection. At the end of this period, the fluid injected was changed so as to consist again of 5% d-glucose. Between 1.1 and 2.5 g of acacia per kilo of body weight has been administered in different experiments. The rates of excretion of creatinine, urea, water and chloride before, during and after the injection of solution of acacia were studied, as well as the hematocrit values and the concentration of the plasma proteins and of acacia in the plasma.

*Results and Comment.* Some of the results of 4 such experiments, comprising 11 periods of observation before the administration of acacia and 32 periods during its infusion and subsequent to it, have been represented in Fig. 1. In general, the experiments show the following features: (1) creatinine clearance remains essentially unchanged; (2) urea clearance shows more fluctuation than creatinine clearance; its larger changes appear definitely associated with changes in urinary volume; (3) urinary volume (water excretion) decreases as a solution of acacia is being infused but shows a tendency to return to its previous levels later in the experiments; (4) the concentration of chloride in the plasma decreases, obviously largely because of the increased volume of the plasma (a consequence of the injection of the salt-free solution of acacia); (5) the rate of excretion of chloride in the urine appears consistently increased after the injection of solution of acacia.

*Chloride Excretion.* The difference between the relative consistency of the creatinine clearance and the rather marked changes in the rate of excretion of chloride is obvious. In experiment D, the increase of the rate of excretion of chloride is the least striking; however, the maintenance of its rate of excretion at the original level appears significant, since the volume of urine excreted per minute following the injection of solution of acacia was diminished by 79%, and the concentration of chlorides in the plasma decreased from an average of 375 to 352 mg per 100 cc.





Glucose 5%, 200 c.c. per hour, in each experiment (intravenously)

FIG. 1.

The influence of acacia on the renal excretion of water, chloride and creatinine (exogenous).

As an average for all the experimental periods, the increase of the rate of excretion of chloride observed amounted to 189%. In experiments B and C there was no overlapping of the values of chloride excretion before and after injection of the solution of acacia. Under the same circumstances, the creatinine clearance showed an average decrease of 3% and in all instances the values before and after the injection of solution of acacia showed appreciable overlapping.

Although the absolute change in terms of clearance of chloride

seems nearly infinitesimal when compared with the magnitude of the volume of the glomerular filtrate, it would seem important nevertheless from the point of view of chloride balance. Physiologically and pathologically, this appears significant, especially since, in the nephrotic syndrome, the excretion of sodium chloride is impaired, and its retention, along with water, gives rise to the pathologic accumulation of edema fluid. These considerations gain added importance, since Binger, Keith, and one of us (Goudsmit)<sup>1</sup> showed comparable increases of the rates of excretion of chloride in patients having nephrotic edema under treatment with solution of acacia.

*Plasma Proteins.* In all experiments, the concentration of plasma proteins was decreased, following the injection of a solution of acacia. This decrease was roughly proportional to the dose of acacia, and appears to have been caused chiefly by dilution, since the change in the concentration of plasma proteins can be rather satisfactorily predicted from the changes in the values of the hematocrit. The deviation of the average predicted change in plasma proteins from the average change observed is 3%. The concentrations of acacia in the plasma observed subsequent to the completion of its injection varied between 1210 and 3100 mg per 100 cc.

*Summary.* In experiments on dogs, after the intravenous injection of acacia, it was found that (1) the rate of glomerular filtration is essentially unchanged; (2) water excretion shows a diphasic response; (3) chloride excretion is markedly increased; (4) plasma proteins and hematocrit values diminish in comparable extent.

### 13103

#### Further Studies on a Frog Method for Assaying Gallbladder-Contracting-Substances.

LLOYD D. SEAGER.

*From the Departments of Pharmacology, St. Louis University and University of Tennessee, Memphis, Tenn.*

Cholecystokinin is usually assayed by the dog method,<sup>1</sup> incompletely described, and by the guinea pig method.<sup>2, 3</sup> Both are time

<sup>1</sup> Goudsmit, Arnoldus, Jr., Binger, M. W., and Keith, N. M., unpublished data.

<sup>2</sup> Ivy, A. C., and Oldberg, E., *Am. J. Physiol.*, 1928, **86**, 599.

<sup>3</sup> Agren, Gummar, *Skand. Arch. J. Physiologie*, 1934, **69**, 1.

<sup>3</sup> Doubilet, H., and Ivy, A. C., *Am. J. Physiol.*, 1938, **124**, 379.

consuming and expensive in animal materials. In a previous communication,<sup>4</sup> I have described a simple frog method of assay and the present report is concerned with factors that may affect it. The influence of sex, season, repeated injections, starvation and pH have been studied. A comparison has been made of the response of the dog and frog to the same product. Two new preparations of cholecystokinin have been made according to Ivy's S.I. method<sup>6</sup> from dog intestine and compared to the standard previously used. The assay is as follows:

Active dark-colored male frogs weighing 20-40 g are selected, cerebra are crushed, cords pithed and viscera exposed through a paramedian incision. The bloodflow to the gallbladder is observed under a low-power microscope and only those preparations are used that show an active circulation. Injections are made intracardially. The criterion of contraction of the gallbladder is the visual observation of a definite rounding, irregularity or opalescence. When more than one injection is made in the same animal, at least 40 minutes are allowed between injections.

A frog unit is defined as the amount of substance that, when injected intracardially per 30 g frog, brings about contraction of the gallbladder in 50% of 20 or more experiments.

The standard used for comparison is a preparation of S.I. (Ivy) used for experiments previously reported<sup>4</sup> and having a potency of 25 frog units per milligram.

*Results and Discussion.* Comparison of the gallbladder response of male and female frogs to S.I. injection was made on early summer frogs. Female frogs appear to be slightly less sensitive than males, giving an assay of 20.8 units per mg as compared to 23.7 units per mg for the males.

An analysis of first and subsequent injections in this group of animals shows no appreciable difference in response to the same dosage. Forty minutes or more were allowed between injections in the same animal.

Some seasonal variation in response to S.I. is apparent. Using the same standard preparation, early spring frogs showed an assay potency of 24.4 units per mg; early summer frogs 23.7 units per mg; normal late summer frogs 10.8 units per mg; and fall and winter frogs 21.7 units per mg. Summer frogs that had been starved at room temperature for 3 months presented gallbladders that were markedly distended with thick bile. There were only a few of the gall-

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<sup>4</sup> Seager, L. D., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 326.

<sup>6</sup> Greengard, H., and Ivy, A. C., *Am. J. Physiol.*, 1938, **124**, 427.

TABLE I.  
Influence of Sex, Season and Starvation on the Assay of S.I.

	% solution	No. of exp.	Contractions	No change
Spring male frogs	.030	40	36	4
	.025	73	55	17
	.020	32	15	17
	.015	29	3	26
Early summer male frogs	.030	20	18	2
	.025	104	72	32
	.020	60	27	33
	.015	32	8	24
Early summer female frogs	.025	98	53	45
	.020	60	26	34
	.015	28	5	23
Starved summer frogs	.025	20	0	20
	.050	24	3	21
	.10	28	4	24
Normal summer frogs	.10	24	15	9
	.05	40	21	19
	.03	24	7	17
Fall and winter frogs	.030	48	38	10
	.025	24	14	10
	.020	28	11	13

bladders of this group of animals that contracted even when large doses of S.I. were used. This finding is in keeping with the observation of McMaster and Elman<sup>5</sup> that with starvation there is a storage and concentration of bile. Failure of these distended bladders to contract is possibly explained by the experiments of Ivy and Doubilet<sup>3</sup> showing that distension of the gallbladder beyond a certain optimal pressure greatly reduced its response to cholecystokinin. Analysis of the above results indicates that with the exception of starved summer frogs and late summer normal frogs, there is no significant statistical seasonal variation.

Two new preparations of S.I. were made from dog duodenum and intestine. S.I.T.<sub>5</sub> was prepared as usual by Ivy's method. S.I.T.<sub>10</sub> was prepared from the filtrate remaining after removal of S.I. from the 5% trichloroacetic acid. When the concentration of this acid is brought up to 8-10%, a new precipitate forms which is much finer in texture and settles out more slowly. It was collected on filter paper (Whatman No. 5), washed with absolute aldehyde-free acetone and ether and dried in a desiccator.

Assay of the two preparations showed a potency of 60 units per

<sup>5</sup> McMaster, P. D., and Elman, R., *J. Exp. Med.*, 1926, **44**, 173.



mg for the S.I.T.<sub>10</sub> and 8.9 units per mg for the S.I.T.<sub>5</sub>. Winter frogs were used.

An attempt was made to rule out histamine-like or choline-like substances as being the active components in these extracts.

0.2 cc of 0.1% atropine sulfate per 30 g frog was injected intracardially in 24 frogs. Subsequent injections of 0.2 cc of 0.1% S.I.T.<sub>5</sub> gave gallbladder contractions in 23 animals. The dosage of atropine was sufficient to partially or completely block the effect of vagus stimulation on the heart.

That an H-like substance is not an active component of the extracts in these experiments is borne out by the fact that 42 intracardiac injections of histamine of doses varying from 0.1 to 0.4 mg produced no contractions.

Frog saline of varying pH, 5 to 9, produced only one contraction in 82 experiments.

Using the dog method of assay,<sup>1</sup> 5 of 11 dogs showed increased gallbladder pressure on the intravenous injection of 0.2 mg of S.I. per kilo. Weight for weight according to this result, the dog is about 16 times as responsive to S.I. as is the frog. The dog, however, is more expensive as assay material and entails much more labor and time.

*Summary.* The frog is a satisfactory animal for the assay of gallbladder contracting materials and has the advantage over others of time and expense. Assays on normal summer frogs give lower figures than at other seasons. There is no significant variation in the assays on spring, early summer, male and female frogs, or winter frogs. Starved frogs have gallbladders greatly distended with thick bile. Such distended bladders do not respond well to S.I. The response of the frog's gallbladder to S.I. is not due to the presence of histamine or choline-like substances in the extracts. A modification of Ivy's method for the preparation of S.I. has resulted in the isolation of a more potent preparation and the recovery of much active material lost by the original procedure.

## 13104 P

## Toxicity of Actinomycin.

SELMAN A. WAKSMAN, H. ROBINSON, H. J. METZGER AND H. BOYD WOODRUFF.

*From the New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, N.J., and the Merck Institute of Therapeutic Research, Rahway, N.J.*

An *Actinomyces* possessing strong antagonistic properties to all the bacteria so far tested and to a number of fungi has been isolated from the soil by two of us and described as *Act. antibioticus*.<sup>1</sup> An active substance was obtained in crystalline form from cultures of this organism grown on organic and inorganic media and designated as actinomycin. This substance has strong bacteriostatic and bactericidal properties.

The following observations are presented here to illustrate the extreme toxicity of actinomycin. In most experiments, the purified crystalline actinomycin A was employed. It was dissolved in alcohol to give 5-10 mg per 1 cc, and diluted further with sterile water or saline solution. The acute toxicity of actinomycin was determined in 320 mice, 60 rats, 19 guinea pigs, 32 rabbits, 3 hens and 50 15-day-old chick embryos. Doses ranging from 0.10 to 30 mg per kg body weight were given intravenously, intraperitoneally, subcutaneously and orally. Observations were made frequently during the first 12 hours and thereafter twice daily for varying additional periods. The results obtained in one group of

TABLE I.  
Acute Toxicity of Actinomycin in Mice.  
10 mice used for each treatment.

Dose, mg/kg	No. dead, after days							
	Intrav.		Intrap.		Subcut.		Oral	
	1	7	1	7	1	7	1	7
0.15	0	0	0	0	0	0	—	—
0.25	0	2	0	2	0	1	—	—
0.50	0	8	0	10	0	10	—	—
1.0	9	10	0	10	0	10	—	—
2.0	10	10	8	10	10	10	0	0
5.0	10	10	10	10	10	10	0	0
10.0	—	—	—	—	—	—	0	3
15.0	—	—	—	—	—	—	0	7
20.0	—	—	—	—	—	—	10	10

<sup>1</sup> Waksman, S. A., and Woodruff, H. B., *J. Bact.*, 1940, **40**, 581; 1941, **42**, in press; *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 609.

experiments on mice are presented in Table I. Similar results were obtained in rats, rabbits and other animals.

It is evident that actinomycin is extremely toxic to experimental animals, being considerably more toxic than gramicidin, tyrocidin, or penicillin. Doses of 1 mg or more per kg were lethal, the toxicity becoming more apparent when observations were extended over a 7-day period. Toxic signs consisting of weakness, languor, anorexia and diarrhea, developed 6-12 hours following actinomycin administration. Gross hematuria occurred frequently in rabbits from 4 to 6 hours after intravenous administration of 1 mg per kg. As intoxication progressed, the nervous system became involved as evidenced by ataxia and tonic convulsions. The immediate cause of death appeared to be respiratory failure, since the heart continued to beat for some time after respiration ceased. Congestion in the lungs, liver, spleen and intestinal viscera was found, the most striking phenomenon being the diminution in the size and weight of the spleen.

The 15-day-old chick embryos tolerated a dose of 1 mg per kg weight. However, this dose was sufficient to prevent their hatching.

Working with strains of *Staph. aureus*, *Cl. welchii*, *Strep. hemolyticus* and Pneumococcus Type I, actinomycin was found to have a powerful bacteriostatic effect. Over a 5-day period, with or without the presence of 10% serum, this bacteriostatic action slowly became bactericidal. Actinomycin appears to be somewhat more effective against pneumococci and streptococci than against staphylococci. Anaerobic bacteria are less sensitive than the above aerobic forms.

The antibacterial action of actinomycin *in vivo* was studied in mice using a mouse virulent strain of Pneumococcus Type I and a hemolytic strain of streptococci Lancefield group A. The infected mice were treated immediately after the inoculation with single doses of 0.25, 0.5 and 1.0  $\mu\text{g}$  of actinomycin intraperitoneally, subcutaneously or orally. A second series of experiments consisted of infecting mice intraperitoneally and treating them orally, subcutaneously or intraperitoneally, every 4 hours, until death, with doses of 0.1 and 0.2  $\mu\text{g}$ . Under these conditions actinomycin afforded little or no protection. In a control group of mice 3  $\mu\text{g}$  of gramicidin afforded complete protection. However, some effect was obtained with actinomycin against infection with *Trypanosoma equiperdum*. It was also found that a dose 50  $\mu\text{g}$  was ineffective in protecting guinea pigs and incubated eggs against a virulent strain of *Brucella abortus*.

A dose of 2.5  $\mu$ g actinomycin per 20 g of animal weight, using mice, rats and chickens, was found to disappear from the circulating blood within a period of 60 minutes. Analysis of the urine indicated that within a period of 6 hours from 10-20% of actinomycin was excreted in the urine of rabbits.

*Summary.* Actinomycin is found to be a powerful bacteriostatic and bactericidal agent *in vitro*. The presence of serum does not diminish the efficacy of this substance. However, no protection is afforded to mice inoculated with cultures of *Streptococcus hemolyticus* or *Pneumococcus* Type I, or to guinea pigs inoculated with *Brucella abortus*. The lack of *in vivo* activity may be due, among other things, to the rapid disappearance of actinomycin from the blood. Actinomycin is extremely toxic to all animal species, death apparently resulting from respiratory failure. Most deaths do not occur until 15-20 hours after actinomycin inoculation.

## 13105

Oxygen Consumption and Growth in Cultures of an Obligate Anaerobe, *Bacteroides vulgatus*.

YALE J. KATZ AND MARTIN E. HANKE.\*

*From the Department of Biochemistry, University of Chicago, Chicago, Illinois.*

Following the observations of Knight and Fildes<sup>1</sup> on *Clostridium tetani*, and of Vennesland and Hanke<sup>2</sup> on *Bacteroides vulgatus*, that these two kinds of anaerobes grow in the presence of controlled and limited tensions of oxygen, provided the  $E_h$  is kept below a certain level, it was of interest to determine whether this growth is characterized by oxygen consumption. The latter authors in fact observed that when cultures of *Bacteroides vulgatus* are exposed to pure  $O_2$ , this gas is consumed at a slow rate, but here the organisms were dying rapidly and this oxygen consumption could hardly be related to growth of the anaerobe. In this study oxygen consumption is measured during growth.

The apparatus and procedure were essentially those of Vennesland and Hanke.<sup>2</sup> A 4-ounce wide-mouth bottle containing 75 cc

\* This work was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

<sup>1</sup> Knight, B. C. J. G., and Fildes, P., *Biochem. J.*, 1930, **24**, 1496.

<sup>2</sup> Vennesland, B., and Hanke, M. E., *J. Bact.*, 1940, **39**, 139.



of 1% glucose nutrient broth is fitted with a rubber stopper which supports 2 platinum electrodes, a glass electrode, a salt bridge to a calomel cell, and inlet and outlet tubes for the circulation of a gas mixture. After inoculation with 2 cc of a 36-48 hour brain-broth culture of *Bacteroides vulgatus*, strain *marino*, a gas mixture of purified nitrogen containing about 2% CO<sub>2</sub> is circulated through the medium, and when, after several hours, the oxidation-reduction potential observed with the shiny platinum electrodes has fallen to about +0.100 volt, the oxygen content of the gas is gradually increased so that the potential remains at an E<sub>h</sub> of about +0.100 volt, never rising above 0.150. After about 12 hours evidence of growth appears in terms of increasing turbidity, and a gradual fall in pH, of about 0.1 pH per hour.

At this time, by means of a closed-system, gas-circulating pump, a mixture of about 6% O<sub>2</sub>, 2% CO<sub>2</sub>, and the rest nitrogen is circulated through the culture at such a rate that the E<sub>h</sub> remains below 0.150 volt and growth continues. The gas-circulating device consists of a 300-cc tonometer with both inlet and outlet tubes leading from and to the culture vessel, with a pair of glass valves, so that the gas can pass only in one direction. A mercury leveling-bulb attached to the bottom of the tonometer which is alternately raised and lowered about 10 cm once every 3 seconds by a windshield-wiper motor provides the pressure changes necessary for the motion of the gas. At suitable times 50 cc samples of gas are withdrawn through a side-arm stopcock for analysis for O<sub>2</sub> and CO<sub>2</sub> by the method of Van Slyke and Sendroy.<sup>3</sup>

Controls were studied in which the potential was maintained above 0.150 volt throughout, by starting early the circulation of an oxygen-containing gas mixture. These controls, which differed in their preparation from the test vessels only in the time at which the circulation of the oxygen-containing gas was started, showed no evidence of growth, that is, no turbidity and no significant pH change. Other observations were made in which the potential was kept below 0.150 volt until growth was well started, and then elevated above 0.150 volt during the circulation of the analyzed gas mixture.

*Results.* Table I gives the result of one typical control and test experiment. Eight observations in 4 experiments where growth occurred while circulating gas mixtures containing 3 to 8% O<sub>2</sub>, showed an average oxygen consumption of 0.17 cc pure O<sub>2</sub> per hour. The control O<sub>2</sub> consumption was 0.01 cc per hour which is just

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<sup>3</sup> Van Slyke, D. D., and Sendroy, J., Jr., *J. Biol. Chem.*, 1932, **95**, 509.

TABLE I.  
Oxidation-reduction Potential, pH, and Oxygen Consumption in Cultures of  
*Bacteroides vulgatus*.

Time, hr	Avg potential, millivolts	pH	$\Delta$ pH per hr	% O <sub>2</sub> in gas	Volume of gas in cc	cc pure O <sub>2</sub> consumed by culture	
						in time interval	per hour
0		6.5					
10.5	210	6.4	.01	9.41			
14.0	170	6.4	.00	9.45	150	(+.06)	(+.02)
25	240	6.3	.01	9.35	100	.10	.01
0		6.5					
4.0	130	6.5	0				
	75		.10				
10.0	75	5.9	.13	6.79			
11.5	80	5.7	.25	6.72	225	.16	.10
13.5	130	5.2	.04	6.36	150	.54	.27
23		4.8		5.63	100	.73	.06

With potential above 150 millivolts, there is no growth, no turbidity, no pH change, and no oxygen consumption.

With potential below 150 millivolts, there is extensive growth, marked turbidity (15 mg dry weight of organisms at end), definite pH change, and oxygen consumption.

within the limit of experimental error. In the experiments where growth was allowed to begin but then was diminished by elevating the potential above 0.150 volt, the O<sub>2</sub> consumption was 0.06 cc per hour.

The dry weight of organisms in cultures showing maximum growth averaged 15 mg and the QO<sub>2</sub> on this basis is 11. This 15 mg represents the final weight at the end of the experiment, and the average weight during the circulation of the gas was undoubtedly less; so the QO<sub>2</sub> of 11 is a minimal value. This value is close to the QO<sub>2</sub> of 6-12 calculated by Stickland<sup>4</sup> from observations on methylene blue reduction in a Thunberg tube by *Clostridium sporogenes*.

*Conclusion.* Although *Bacteroides vulgatus* grows in the absence of oxygen and is killed by air, its growth at low oxidation-reduction potential in the presence of 3 to 8% oxygen is characterized by oxygen consumption.

<sup>4</sup> Stickland, L. H., *Biochem. J.*, 1934, **28**, 1746.

## 13106 P

## Inactivation of Estrone in Normal Adult Male Rats.

GERSON R. BISKIND.

*From the Department of Pathology, Mount Zion Hospital, San Francisco, and the Division of Pathology, University of California Medical School, San Francisco.*

Previous observations have shown that estrogens and androgens are inactivated by the liver in castrate rats of the appropriate sex,<sup>1, 2</sup> and that normal female rats inactivate testosterone propionate.<sup>3</sup> Estrogens and androgens have been demonstrated to be present in both sexes.<sup>4</sup> It has recently been found that of all tissues hitherto examined the greatest concentration of estradiol is in the testes of horses.<sup>5</sup> These facts indicate that a system must be present to inactivate the estrogens occurring in males. The following experiment was designed to investigate this problem.

The technic employed in this experiment was similar to that previously described.<sup>3</sup> The pellets of estrone (keto-hydroxy-estra-triene\*) were prepared in a mold and uniformly compressed. Normal adult male rats of the hooded type were employed. In one group the pellet was implanted in the subcutaneous tissues; in another group in the spleen. In a third group it was implanted in the spleen, and this organ was subsequently transplanted between the skin and subcutaneous tissues, and after an interval of 16 days its vessels were ligated. The right testis was removed in half the number of animals of each group at the time the pellet was implanted to provide an additional control. The animals were sacrificed 42 days after insertion of the pellets, and the organs were quickly dissected and immediately weighed on a damped balance. A group of 8 normal adult male rats served as controls.

The results are shown in Table I, which indicates the site of the pellet, the number of animals in each group, the average weight of the animals and the average weights of the right testis, left testis, bulbo-urethral muscles, seminal vesicles and prostate, respectively for each group. The average amount of estrone absorption in each group is also shown, determined by weighing the pellet at the beginning and at the termination of the experiment.

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<sup>1</sup> Biskind, G. R., and Mark, J., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 212.

<sup>2</sup> Biskind, G. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 259.

<sup>3</sup> Biskind, G. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1941, **46**, 452.

<sup>4</sup> Gustavson, R. G., In *Sex and Internal Secretions*, Chap. xiv, 1939.

<sup>5</sup> Beall, D., *Biochem. J.*, 1940, **34**, 1293.

\* Supplied through the courtesy of Parke, Davis & Co., Detroit, Mich.

TABLE I.

Location of pellet	No. of animals	Avg body wt, g	Right testis, mg	Left testis, mg	Bulbo-urethral muscles, mg	Prostate, mg	Seminal vesicles, mg	Avg daily absorption 42 days, mg
None	8	460	1721	1696	507	1024	506	—
Spleen	4	434	1485	1566	512	1205	439	.016
"	4	475	1687*	1921	539	1276	539	.016
Transplanted spleen	3	335	362	367	191	468	190	.012
"	3	313	1563*	370	169	358	163	.016
Subcutaneous tissues	4	285	273	283	139	276	139	.023
"	4	279	1600*	358	147	332	146	.012

\*Right testis removed at time of implantation of pellet.

A pellet of estrone implanted in the subcutaneous tissues of a normal adult male rat produced marked atrophy of the testes. This effect was mediated by the inhibition of the hypophyses with suppression of the gonadotropic hormone.<sup>6</sup> When the pellet was implanted in the spleen, permitting the absorbed material to be transported to the liver by way of the portal system, the specific effect was not evident. Transplantation of the spleen and ligation of its vessels diverted the absorbed hormone into the systemic circulation and produced results similar to those produced by the pellet in the subcutaneous tissues. This excluded the spleen as the site of inactivation.

Removal of one testis caused slight hypertrophy of the remaining testis. In the groups in which one testis had been removed at the time of implantation of the pellets and the remaining testis had undergone atrophy, the latter was of slightly greater average weight than in the groups in which both testes atrophied simultaneously. Thus, where there was but one testis there was no greater degree of atrophy; therefore confirming the observation of Hertz and Meyer.<sup>7</sup>

The prostates, bulbo-urethral muscles and seminal vesicles showed diminution in weight in the groups that suffered testicular atrophy. These changes were probably due both to loss of specific testicular stimulation, and to the direct effect of estrone.<sup>8</sup> The histologic changes in all the organs will be described in a subsequent publication.

*Summary and Conclusions.* Pellets of estrone were implanted in the subcutaneous tissues, the spleen, and the transplanted ligated spleen, respectively, of groups of normal adult male rats. At the

<sup>6</sup> Meyer, R. K., Leonard, S. L., Hisaw, F. L., and Martin, S. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1930, **27**, 702.

<sup>7</sup> Hertz, R., and Meyer, R. K., *Am. J. Physiol.*, 1938, **124**, 259.

<sup>8</sup> Freud, J., *Biochem. J.*, 1933, **27**, 1438.



time of implantation of the pellet the right testis was removed in half the number of animals of each group. Marked atrophy occurred in the testes and related genital organs when the pellet was present in the subcutaneous tissues or in the transplanted ligated spleen. No changes occurred in these organs when the pellet was present in the normally situated spleen, indicating that when the absorbed estrone passes through the liver of normal adult male rats before reaching the organs it specifically affects, it is inactivated.

The technical assistance of Ruth Helmuth is gratefully acknowledged.

### 13107 P

#### Death of Embryos in Guinea Pigs on Diets Low in Vitamin E.

ALWIN M. PAPPENHEIMER AND MARIANNE GOETTSCH. (With the assistance of Claudia Schogoleff.)

*From the Departments of Pathology and of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York.*

The need of vitamin E for the successful completion of pregnancy in rats is well known.<sup>1</sup> More recently it has been shown to be essential in mice also.<sup>2, 3</sup> We have found no reference in the literature describing resorption of the embryo due to vitamin E deficiency in guinea pigs. The failure to demonstrate this heretofore has been due undoubtedly to the fact that guinea pigs on vitamin E-low diets usually die of muscular dystrophy before the onset of sexual maturity.

It seems worthwhile, therefore, to place on record certain observations which indicate that the guinea pig, like the rat and mouse, requires an abundant supply of vitamin E, and that an inadequate intake results in death of the embryo *in utero*.

*Experimental.* The basal diet V consisted of:

Casein (commercial)	200
Sucrose	150
Cornstarch	360
Yeast	50

<sup>1</sup> Evans, H. M., and Burr, G. O., *Memoirs of the Univ. of Cal.*, 1927, No. 8.

<sup>2</sup> Bryan, W. L., and Mason, K. S., *Am. J. Physiol.*, 1940, **131**, 263.

<sup>3</sup> Goettsch, M., and Pappenheimer, A. M., *Proc. Am. Inst. Nutrition*, 1941, Chicago.

Salt mixture	40
Lard	80
Cod liver oil	20
Cellulose	200

This was supplemented by 5 cc of tomato juice and 25 g of fresh lettuce daily. Lettuce was added to supply a necessary water soluble growth factor, the need for which in guinea pig nutrition has been indicated by the work of Kohler, Elvehjem and Hart,<sup>4</sup> and of Cannon and Emerson.<sup>5</sup> The amount of vitamin E contained in this amount of lettuce has been found by us in previous experiments inadequate to protect against muscle dystrophy.

Of 3 controls on this basal diet, 2 died after 101 and 312 days with severe muscular dystrophy. The third died at 286 days without muscle lesions.

Confirming our previous experiences, the addition of 25 g of lettuce daily did not provide sufficient vitamin E to protect 2 of the 3 animals against muscular dystrophy, nor to ensure a successful pregnancy.

Fifteen guinea pigs received a weekly supplement of 5 or 10 mg of alpha-tocopherol acetate (Hoffman-LaRoche). Ten of these had to be discarded from the experiment for various reasons. A brief history of the remaining 5 follows:

*Guinea Pig No. 63*, receiving 5 mg of alpha-tocopherol weekly, was operated upon on the 30th day after mating, having had a bloody vaginal discharge for 2 days, and a partially resorbed fetus removed from the right uterine horn. This animal is alive and has again been mated, receiving 25 mg of alpha-tocopherol, 2 days after being placed with the male.

*Guinea Pig No. 47*, which also received 5 mg of alpha-tocopherol weekly, was mated unsuccessfully 6 times. Twenty-nine days after the 7th mating a bloody vaginal discharge was observed. An operation, which resulted in death on the following day, disclosed 4 dead fetuses, 28, 30, 25, and 20 mm in length.

*Guinea Pig No. 54*, also receiving 5 mg of alpha-tocopherol weekly, became pregnant after her 5th mating. Bloody vaginal discharge was noted 26 or 27 days after mating, and the animal was sacrificed. One living fetus 15 mm in length, was found in the left uterine horn, and a dead fetus, partly macerated, in the right horn.

*Guinea Pig No. 46*, receiving 10 mg weekly, became pregnant after the first mating. It was found dead on the 47th day; at autopsy,

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<sup>4</sup> Kohler, G. O. Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, 1938, **15**, 445.

<sup>5</sup> Cannon, M. D., and Emerson, G. A., *J. Nutrition*, 1939, **18**, 155.

2 well preserved fetuses, which microscopically showed subcutaneous and intramuscular oedema, were present in the uterus.

*Guinea Pig No. 52*, received 10 mg weekly. Sixty days after the first mating, it gave birth to a single healthy infant which survived for 53 days without becoming dystrophic, and died from traumatic accident. The mother was again mated; 33 days after being placed with the male, a bloody vaginal discharge lasting 2 days was noted. Probably resorption took place at this time, but the presence of a dead fetus was not confirmed by operation.

Thus, in these 5 guinea pigs, the tocopherol protected the animals against muscular dystrophy, but the amount was not adequate to insure successful pregnancy. Those receiving 5 mg had resorption at about 30 days, or midway in the term of pregnancy. Of the 2 receiving 10 mg, one gave birth to a living young, followed by resorption (initial fertility); the other went considerably beyond mid-term, dying on the 47th day.

It will be necessary, before definitely ascribing the fetal deaths to vitamin E deficiency, to carry the experiments further, and to establish the minimal dose of alpha-tocopherol required for successful completion of pregnancy. It may be said provisionally, however, that on a low E diet, supplemented by lettuce and weekly doses of 5-10 mg, resorption of the embryos does occur in a high proportion of cases. This amount is sufficient to prevent muscular dystrophy.

Examination of the dead fetuses has not given a clear explanation of the cause of death. Aside from oedema and early autolytic changes, nothing of significance was detected. Sections of the placenta of the living fetus of guinea pig No. 54 showed extensive areas of hemorrhagic and anemic necrosis; the placenta of the dead fetus, and that of the other fetuses, were the seat of complete hemorrhagic necrosis. It will be necessary to have further observations before concluding that the placental lesions are of primary importance.\*

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\* We are greatly indebted to Dr. Shaner of the Hoffman-LaRoche Company for the dl-alpha-tocopherol acetate used in these experiments.

### Relation of Cystine to Achromotrichia.

P. L. PAVCEK<sup>†</sup> AND H. M. BAUM. (Introduced by W. H. Griffith.)

*From the Biological Laboratories, Anheuser-Busch, Inc., St. Louis, Mo.*

It has been shown by Mulford and Griffith<sup>1</sup> that the cystine furnished by 18% casein as the sole source of dietary protein may be a limiting factor in the growth of the rat. Since cystine has been investigated<sup>2, 3, 4</sup> in relation to hair growth in the rat and found to influence the amount of hair produced, the study of the relation of this amino acid to nutritional achromotrichia was undertaken.

Weanling black male rats of the Wisconsin strain between the ages of 18 and 23 days and weighing 40-45 g were placed on a diet consisting of starch (Anheuser-Busch) 62%, Labco casein 18%, Crisco 14%, salt mixture (Merck No. 1) 4%, and cod liver oil (Mead Johnson) 2%. Supplements of 25  $\gamma$  thiamine, 20  $\gamma$  riboflavin, 20  $\gamma$  pyridoxine, 300  $\gamma$  nicotinic acid and 5 mg choline\* were fed daily to each animal.

On the basal diet the rats became grey in 4-5 weeks and plateaued in weight by the 6th week. Administration of 200  $\gamma$  pantothenic acid (as calcium salt) daily to a group of 10 animals resulted in marked diminution in greying. The blackening of the fur began in 4-5 weeks after the beginning of pantothenic acid administration and reached a maximum intensity only after 12-14 weeks. It should be pointed out that the regeneration of black fur was incomplete—the coat being interspersed with grey resulting in a stippled effect much like that described by Dimick,<sup>5</sup> Williams,<sup>6</sup> and Unna.<sup>7</sup> A group of 7 animals receiving daily 75 mg cystine in addition to the pantothenic acid supplement reached the same final stage of the regeneration in but 5-7 weeks. In this instance the first darkening of the fur appeared in 3-4 weeks. It was interesting that during these first few weeks the rats receiving only pantothenic acid exhibited

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<sup>†</sup> Present address: St. Louis University School of Medicine, St. Louis, Missouri.

<sup>1</sup> Mulford, D. J., and Griffith, W. H., Abstracts 101st Meeting of A.C.S., St. Louis, Mo., April 7-11, 1941.

<sup>2</sup> Lightbody, H. D., and Lewis, H. B., *J. Biol. Chem.*, 1929, **82**, 485.

<sup>3</sup> Beadles, J. R., Braman, W. W., and Mitchell, H. H., *Ibid.*, 1930, **88**, 623.

<sup>4</sup> Smuts, D. B., Mitchell, H. H., and Hamilton, T. S., *Ibid.*, 1932, **95**, 283.

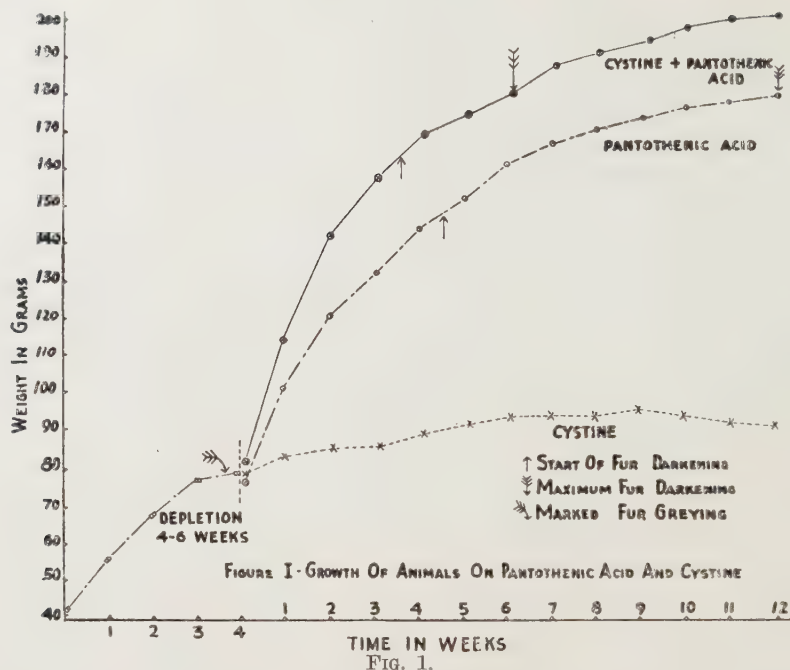
\* Increased to 20 mg in the added cystine groups.

<sup>5</sup> Dimick, M. K., and Lepp, A., *J. Nutrition*, 1940, **20**, 413.

<sup>6</sup> Williams, R. R., *Science*, 1940, **92**, 561.

<sup>7</sup> Unna, K., and Sampson, W. L., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 309.





intensification of the greying, whereas the group receiving cystine in addition did not show this phenomenon. The third group of 4 animals on cystine alone became increasingly grey.

The responses in growth on the various supplements is illustrated graphically in Fig. 1. It is evident that the rats receiving both pantothenic acid and cystine grew faster than those on pantothenic acid alone. During the first 5 weeks the former group averaged almost 4 g per week more than the latter. Even after the animals had been on the supplements for 14 weeks, the cystine-pantothenic acid animals were over 20 g heavier than those receiving only pantothenic acid.

*Summary.* From the above data it appears that on an 18% casein diet, the supplementation of 75 mg of cystine in addition to 200  $\gamma$  of pantothenic acid per rat per day markedly decreases the time required for the replacement of the grey hair of nutritional achromotrichia. Since better growth results when this amount of cystine is administered, the findings of Mulford and Griffith<sup>1</sup> that 18% casein diets do not furnish enough cystine for maximum growth of the rat, are substantiated.

The authors wish to acknowledge the technical assistance of Mr. Alois E. Rempe in this work.

## 13109

## Influence of Sex on the Evolution of a Transplantable Mouse Sarcoma.\*

LUDWIK GROSS. (Introduced by H. Goldblatt.)

*From the Institute for Medical Research, Christ Hospital, Cincinnati, Ohio.*

While the relation between sex and the development of certain spontaneous tumors in mice has been established definitely, there is no convincing evidence of the influence of sex on the evolution of implanted tumors. This report concerns observations which show that under certain specific conditions there is a distinct difference in the behavior of a transplantable sarcoma in male and female mice.

The possibility that a transplantable sarcoma behaves differently in male than in female mice was suggested by our recent experiments performed in this laboratory in which small doses of sarcoma S-37 were injected intradermally. In a small series of animals it was observed incidentally that the occurrence of *takes*, as well as the mortality rate due to the tumor growth, was higher in males than in females. More detailed experiments were undertaken to determine whether this observation could be substantiated in systematic studies.

*Experimental. Material and Methods. Tumor:* The transplantable mouse sarcoma S-37<sup>†</sup> was used in our experiments. A subcutaneous tumor, 10 to 14 days old, free from necrotic material, was removed aseptically, weighed, and placed in a sterile mortar. The tumor tissue was cut into small pieces with scissors and then ground thoroughly in a mortar, 0.85% solution of sodium chloride being added to obtain suspensions which varied from 1 to 30%. After 5 to 10 minutes of grinding, the suspension was passed through a fine, 56 to 60 mesh screen having wires 0.01 of an inch in diameter.

*Inoculation:* 0.02 to 0.04 cc, and occasionally more of this cell suspension was injected intradermally into the freshly shaved skin in the middle of the back of the mouse, a tuberculin syringe and 27-gauge needle being used for the injection. A small wheal appeared in the skin following each inoculation. Intradermal inoculations were employed exclusively, because our previous studies with

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\* Aided by a grant from Mrs. Francis F. Prentiss.

† We are indebted to Dr. H. B. Andervont, National Cancer Institute, Bethesda, Maryland, for this tumor strain.

Besredka<sup>1-4</sup> showed that the resistance of the mouse to tumor implantation can be demonstrated much better following intradermal inoculation than following inoculation by other routes—an observation which has been confirmed by Andervont.<sup>5</sup>

Several successive series of experiments were performed. In each, groups of male and female mice were injected intradermally with equal amounts of the same tumor-cell suspension. Adult, white mice weighing 18 to 24 g, all of the same stock strain, were used.

*Results. Incidence of Takes:* Within 5 to 15 days, in most cases, however, within the first week following inoculation, small spherical growths (microscopically typical sarcomata) appeared in the skin of the mice. Table I shows that the incidence of *takes* was substantially higher in males than in females, when diluted tumor-cell suspensions were inoculated.

This difference in the resistance of the males and females toward

TABLE I.  
Incidence of "Takes" Using Small Doses of Mouse Sarcoma S-37.

Exp. No.	Tumor suspension		Sex	No. of mice	No. of "takes"	% "takes"
	% conc.	cc inject.				
1	1	.03-.04	♂	18	5	28
			♀	20	2	10
2	2.5	.02-.03	♂	20	5	25
			♀	20	1	5
3	5	.03-.04	♂	13	8	61
			♀	15	7	47
4	5	.02-.03	♂	18	11	61
			♀	20	5	25
5	5	.02-.03	♂	17	16	94
			♀	21	12	57
6	5	.03-.04	♂	10	4	40
			♀	9	0	0
7	10	.03-.04	♂	14	14	100
			♀	14	5	36
8	10	.03-.04	♂	13	11	85
			♀	18	10	56
9	10	.02-.03	♂	21	20	95
			♀	18	18	100
10	10	.02-.03	♂	20	12	60
			♀	20	9	45
11	15	.03-.04	♂	18	17	95
			♀	16	13	81
Total: Exp. 1 to 11			males	182	123	68
			females	191	82	43

<sup>1</sup> Besredka, A., and Gross, L., *Compt. rend. Acad. d. sc.*, 1935, **200**, 175.

<sup>2</sup> Besredka, A., and Gross, L., *Compt. rend. Acad. d. sc.*, 1935, **200**, 790.

<sup>3</sup> Besredka, A., and Gross, L., *Ann. Inst. Pasteur*, 1935, **55**, 402.

<sup>4</sup> Besredka, A., and Gross, L., *Ann. Inst. Pasteur*, 1935, **55**, 491.

<sup>5</sup> Andervont, H. B., *Pub. Health Rep.*, 1937, **52**, 1885.

TABLE II.  
Incidence of "Takes" Using Medium and Large Doses of Mouse Sarcoma S-37.

Exp. No.	Tumor suspension		Sex	No. of mice	No. of "takes"	% "takes"
	% conc.	cc inject.				
12	20	.03-.04	♂	13	13	100
13	20	.03-.04	♀	15	15	100
			♂	11	11	100
14	20	.03-.04	♀	15	13	87
			♂	9	9	100
15	20	.02-.03	♀	9	9	100
			♂	20	20	100
16a	20	.02-.03	♀	21	21	100
			♂	20	20	100
16b	30	.10	♀	22	21	96
			♂	13	13	100
17	25	.20	♀	13	13	100
			♂	18	18	100
			♀	18	18	100
Total: Exp. 12 to 17			males	104	104	100
			females	113	110	98

intradermal implantation of this tumor was discernible only when the more dilute suspensions of tumor cells were inoculated. As is shown in Table II, there was practically no difference in the incidence of *takes* in males and females when the more concentrated suspensions were inoculated.

*Incidence of Spontaneous Regression:* The cutaneous tumors produced by intradermal inoculation increased rapidly in size. Usually within 10 to 12 days after inoculation they became red and shiny. At that time they measured 6 to 9 mm in diameter, except when massive doses were inoculated, in which instance the tumors were larger.

In some instances these cutaneous tumors continued to increase in size and finally killed their hosts within 28 to 80 days following inoculation. The average survival time of the male animals was shorter than that of the females.

In other instances, as has been observed previously,<sup>1-4</sup> the tumors disappeared spontaneously within 15 to 30 days following inoculation. The incidence of spontaneous disappearance of these tumors was substantially higher in females than in males in all series where either small or medium-sized doses of tumor-cell suspensions were inoculated (Table III). However, this difference in the incidence of tumor regression in males and females was less striking when very large doses of tumor-cell suspensions were inoculated. This is shown in Experiment 17, and especially in Experiment 16, Table III, in which one group of male and female mice received 0.02 to 0.03 cc



of a 20% cell suspension and another group received 0.10 cc of a 30% suspension of the same tumor. In the first group, the incidence of spontaneous tumor regression was 65% in females as compared with 12% in males. In the second group, in which large doses were inoculated, the incidence of regression was 23% and 8% for the females and males, respectively.

*Summary.* Experiments reported in this paper suggest that female mice are more resistant than males to an intradermal implantation of a small dose of a mouse sarcoma, as evidenced by the smaller incidence of *takes* and greater incidence of regression in the female animals.

TABLE III.  
Incidence of Spontaneous Regression.

Exp. No.	Tumor suspension		Sex	No. of mice with tumors*	No. of mice tumors regressed	% regression
	% conc.	cc inject.				
1	1	.03-.04	♂	5	2	40
3	5	.03-.04	♀	2	1	50
			♂	4	1	25
4	5	.02-.03	♀	6	5	83
			♂	11	1	9
5	5	.02-.03	♀	3	1	33
			♂	16	2	12
7	10	.03-.04	♀	12	7	58
			♂	14	3	21
8	10	.03-.04	♀	5	4	80
			♂	10	3	30
9	10	.02-.03	♀	9	5	55
			♂	20	4	20
10	10	.02-.03	♀	17	9	53
			♂	12	1	8
11	15	.03-.04	♀	9	3	33
			♂	17	3	18
12	20	.03-.04	♀	11	5	45
			♂	11	1	9
13	20	.03-.04	♀	15	6	40
			♂	11	1	9
14	20	.03-.04	♀	13	5	38
			♂	8	2	25
15	20	.02-.03	♀	8	6	75
			♂	20	2	10
16a	20	.02-.03	♀	21	11	52
			♂	17	2	12
16b	30	.10	♀	20	13	65
			♂	13	1	8
17	25	.20	♀	13	3	23
			♂	18	2	11
			♀	18	5	28
Total of all except 16b and 17:			males	176	28	16
			females	151	81	54

\*The numbers of mice in some of the experiments in this table are smaller than those reported in the corresponding experiments in Tables I and II, because some of the mice reported in Tables I and II were sacrificed for microscopic studies of the produced tumors and therefore eliminated from further observation.

## 13110

**Birefringence and Contractile Power of Muscles During Atrophy Due to Upper Neurone Lesions or Tenotomy.**

ERNST FISCHER.

*From the Department of Physiology and Pharmacology, Medical College of Virginia, Richmond.*

In a recent paper Solandt and Magladery<sup>1</sup> reported that rat muscles deprived of the upper motor neurone control, atrophy to about half of their original weight during the first 2 weeks, but that thereafter the spastic muscles regain weight to a considerable extent. About 1½ years ago, I made similar observations when trying to determine during muscular atrophy due to section of the upper neurones or to tenotomy, the relation between birefringence and contractile power (direct stimulation with currents of optimal frequency and duration). The cumbersome method of the determination of birefringence restricted my experience to a very limited number of rats, and, since the secondary gain in weight of the spastic muscles threw some doubt upon the completeness of the elimination of the upper neurone control, I did not publish my results. Since the correctness of this secondary weight gain has been confirmed now on much more material with sufficient controls, my results concerning the contractile power during atrophy due to upper neurone lesions have gained in significance. The methods employed were those reported for the investigation of the atrophy of denervated rat gastrocnemius-soleus muscles.<sup>2</sup> The elimination of the upper motor neurone control was accomplished either by semi-section of the spinal cord in the region of the fifth or sixth vertebra or by total section of the cord at this level. In the latter case, the sciatic nerve of the opposite side was resected simultaneously in order to enable a direct comparison between upper neurone and final neurone atrophy. In the experiments with atrophy due to tenotomy, the *os calcis* was clipped off and sutured to the skin.<sup>3</sup> The tables of this paper contain for comparison purposes some of the data found for denervation atrophy and previously published.<sup>2</sup> Table I gives the data for atrophies of 9 to 10 days' duration. For all 3 types of atrophy, there exists no diminution in contractile power per weight unit and no diminution in birefringence, despite the fact that the

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1 Solandt, D. Y., and Magladery, J. W., *Proc. Am. Physiol. Soc.*, 1941, p. 268.

2 Fischer, E., *Am. J. Physiol.*, 1940, **131**, 156.

3 Hines, H. N., and Knowlton, G. C., *Am. J. Physiol.*, 1940, **128**, 97.

TABLE I.  
Comparison of Left Muscle with Right Muscle of the Same Rat.

Exper. procedure	Days after operation	Wet wt		Tension		Gram tension per g wet wt	Total birefringence	
		Right g	Left g	Right g	Left g		Right 10-3	Left in % of right
Left hemisection spinal cord	9	1.465	1.058	1550	1100	98.6	2.37	102.7
	10	2.466	1.803	2250	1700	103.6	2.54	101.3
Total section spinal cord and left sciatic nerve	10	1.877	1.656	1850	1600	98.3	2.58	97.3
	10	2.080	1.635	2050	1650	100.6	2.23	99.6
Tenotomy of left Achilles tendon	9	2.661	2.132	2300	1850	100.7	2.35	98.5
	10	2.055	1.403	2150	1550	105.2	2.43	101.3
Section of left sciatic nerve	9	2.929	2.060	2350	1800	109.1	2.26	101.8
	10	1.971	1.109	2350	1350	104.2	2.61	97.6

atrophy as manifested by weight loss is distinct. This weight diminution is larger for denervation atrophy than for upper neurone atrophy, while tenotomy results in a weight loss as considerable as that by denervation. The results obtained 21 to 35 days after operation are given in Table II. The weight losses have become more distinct for denervation and tenotomy atrophy, while in the experiments with upper neurone lesions there is apparently a regain of

TABLE II.  
Comparison of Left Muscle with Right Muscle of the Same Rat.

Exper. procedure	Days after operation	Wet wt		Tension		Gram tension per g wet wt	Total birefringence	
		Right g	Left g	Right g	Left g		Right 10-3	Left in % of right
Left hemisection spinal cord	21	2.432	1.882	2300	1750	98.4	2.33	98.3
	28	1.769	1.385	2150	1650	98.3	2.41	100.2
	35	2.226	1.913	1950	1700	101.5	2.38	97.0
Total section spinal cord and left sciatic nerve	27	1.922	1.198	1950	850	70.1	2.35	62.3
	32	2.045	0.868	2050	600	68.8	2.61	58.7
	34	1.783	0.675	1950	500	67.7	2.46	68.4
Tenotomy of left Achilles tendon	23	1.933	1.035	1850	1000	96.5	2.21	96.5
	28	2.567	1.223	2350	1150	102.8	2.38	98.3
	32	2.278	1.185	2250	1100	98.7	2.45	98.7
Section of left sciatic nerve	23	2.056	0.804	2100	550	67.0	2.43	71.3
	29	1.906	0.621	2150	500	70.5	2.47	67.0
	33	1.793	0.561	1850	350	59.9	2.34	63.3

weight. Only in the case of denervation atrophy, the contractile power per weight unit shows a diminution, and this loss in power corresponds roughly to the simultaneously observed diminution in birefringence. This parallelism between contractile power per weight unit and birefringence can serve as a further indication that the submicroscopic crystalline structure of the muscle is essential for the contractile mechanism.

*Summary.* Although in muscular atrophy, either due to final or upper neurone lesions, or due to tenotomy, there is a distinct weight loss after 9 to 10 days, the muscles have at that time still the same contractile power per weight unit and the same birefringence as normal muscles. But 3 to 5 weeks after onset of the atrophy, only muscles atrophying due to upper neurone lesions or to tenotomy have still the contractile power per weight unit and the birefringence of normal muscle. However, for denervated muscle, contractile power and birefringence are distinctly diminished at that time. Only in the latter type of atrophy does the submicroscopic crystalline structure, so essential for the contraction process, become seriously impaired.

### 13111 P

#### Excretion of Pregnandiol in Women with Virilism.

UDALL J. SALMON, SAMUEL H. GEIST AND A. AUSTIN SALMON.  
*From the Laboratories, Mount Sinai Hospital, New York City.*

Butler and Marrian<sup>1</sup> in reporting the isolation of a steroid compound pregnane-3-17-20-triol, from the urine of 2 women with virilism caused by "enlarged adrenals," also noted the presence of "appreciable amounts of pregnandiol." Venning, Weil and Browne<sup>2</sup> later reported the finding of considerable amounts of sodium pregnandiol glucuronidate (12 mg to 30 mg per day) in 2 cases of virilism, one of which was subsequently found to have an adrenal cortex carcinoma and, the other, adrenal cortex hyperplasia.

During the past 2 years, we have been performing pregnandiol excretion studies in women with the arrhenomimetic (virilism) syndrome. In the present communication, we wish to report the results of these studies.

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<sup>1</sup> Butler, G. C., and Marrian, G. F., *J. Biol. Chem.*, 1937, **119**, 565; *Nature*, 1938, **142**, 400.

<sup>2</sup> Venning, E. H., Weil, P. G., and Browne, J. S. L., *J. Biol. Chem.*, *Scien. Proc.*, 1939, **128**, CVII.



*Methods and Materials.* A total of 7 cases was studied. These consisted of 2 cases proven by operation to be adrenal cortex carcinomas and 5 cases of amenorrhea and hypertrichosis of undetermined etiology. Sodium pregnandiol glucuronidate determinations<sup>8</sup> were performed on single 24-, 48-, or 72-hour specimens, for periods varying from 6 to 32 days.

*Results. Pregnandiol Excretion in Patients with Adrenal Cortex Carcinoma.* In the 2 patients in whom adrenal cortex carcinomas were found, the pregnandiol excretion varied in one from 9 to 20 mg per day and, in the other, from 12 to 18 mg per day. In one patient (I.E.), in whom it was possible to obtain post-operative urines, these were found to contain no detectable amount of pregnandiol after removal of the tumor.

*Pregnandiol Excretion in Patients with Arrhenomimetic Syndrome (without adrenal cortex carcinoma).* In the 5 cases in this group, X-rays of the adrenals after intravenous pyelogram and peri-renal insufflation failed to reveal any evidence of adrenal tumor or enlargement. Two of these (E.W. and T.S.) were explored surgically without revealing any evidence of adrenal neoplasm. In all of these, no pregnandiol was found in the urine.

*Discussion.* The 5 cases of virilism who did not excrete pregnandiol have been under observation for periods varying from 6 months to 3 years, without any clinical manifestations suggesting adrenal, ovarian or pituitary neoplasm. On the basis of the stationary clinical picture in these cases, it seems justifiable to assume that the syndrome is not due to a neoplasm but rather to a functional disturbance involving the adrenal cortex.

In contrast to these, the 2 cases with proven adrenal cortex carcinoma had consistently high pregnandiol excretion. Comparable amounts of pregnandiol are found only in pregnancy or during the corpus luteum phase of a normal menstrual cycle. Furthermore, in one patient in whom studies were performed after removal of the tumor, the pregnandiol disappeared from the urine.

It appears from these studies that the excretion of pregnandiol in appreciable amounts (9 to 20 mg daily) in women with the virilism syndrome, in the absence of pregnancy, indicates hyperactivity of the adrenal cortex which could be due to hyperplasia or carcinoma of the adrenal cortex. The excretion of pregnandiol may be of diagnostic value to differentiate virilism caused by adrenal cortex hyperplasia or neoplasia from other types.

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<sup>8</sup> Venning, E. H., *J. Biol. Chem.*, 1937, **119**, 473.

## 13112 P

Serum Protein Regeneration Following Use of Amino Acids\*  
in Nephritis (Nephrotic Stage).

WILLIAM J. MESSINGER. (Introduced by J. Murray Steele.)

*From the Third (New York University) Medical Division of Welfare Hospital,  
Welfare Island, New York.*

During the past few years the effect of administration of solutions of amino acids upon the regeneration of serum proteins in hypoproteinemic individuals has been studied in a variety of cases.<sup>1, 2, 3</sup> That amino acid solutions can maintain nitrogen balance is clear<sup>4</sup> but that regeneration of serum proteins follows their use is uncertain. In hypoproteinemia associated with the "nephrotic stage" of chronic nephritis, evidence for the regeneration of serum proteins following the use of amino acids is lacking.

Among a number of individuals with hypoproteinemia to whom intravenous amino acids were administered at this hospital, there were 2, exhibiting edema, albuminuria and elevation of blood cholesterol, in the "nephrotic stage" of chronic nephritis.

*Procedure.* Both patients were placed upon a 120 g protein diet, low in fat for 2 or more weeks before observations were begun. They were weighed daily. In the first case the period prior to administration of amino acids lasted 7 days, and in the second, 12. Total urinary nitrogen, serum proteins, and occasionally fecal nitrogen were determined by the micro-kjeldahl method.

Both cases had been followed closely for more than a year prior to the present studies. Despite high protein diets and frequent administration of large amounts of iron, the protein levels of the serum and the degree of anemia did not exceed the limits given (Table I). The first case was known to have suffered from nephritis for 5 years and to have exhibited the so-called "nephrotic stage" for 3. In the second case, the onset of nephritis is unknown, but marked albu-

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\* We are indebted to Frederick Stearns & Co. for a generous supply of amino acid solutions made by enzymatic hydrolysis of casein, approximately 80% complete. The amino acids in the hydrolysate were said to be present in the same concentration as in native casein.

<sup>1</sup> Elman, Robert, and Weiner, D. O., *J. A. M. A.*, 1939, **112**, 796.

<sup>2</sup> Elman, Robert, *Ann. Surg.*, 1940, **112**, 4.

<sup>3</sup> Ravdin, I. S., Stengel, Alfred, Jr., and Prushankin, Mitchell, *J. A. M. A.*, 1940, **114**, 107.

<sup>4</sup> Shohl, Alfred T., Butler, Allan M., Blackfan, Kenneth D., and MacLachlan, Elsie, *J. Pediat.*, 1939, **15**, 469.

TABLE I.  
Comparison of Data in Two Cases of Chronic Nephritis in "Nephrotic Stage."

	Case I	Case II
Age and sex	24, female	40, male
Blood pressure (range), mm Hg	140 160 96 110	130 160 94 104
Urea clearance, % normal	30	28
Maximal specific gravity of urine	1.012	1.013
Albumin, g/24 hr in urine	7-10	6-12
Serum protein, g %	3.9-4.8	4.0-4.8
Serum cholesterol, mg%	400-1000	450-600
Hemoglobin (Sahli), g/100 cc	9-11	11
Red blood corpuscles, millions/mm <sup>3</sup>	2.75	2.75

minuria has been present for at least 2 years. Edema has always been much more marked in the first case.

In Case I 500 cc of a solution of 5% amino acids and 5% glucose were injected intravenously daily for 14 days. The injections were given slowly during a period of 3 hours. Increase in level of serum proteins did not occur (Fig. 1).

In the second case, daily intravenous injections of 180 cc of 20% solution of amino acids diluted with an equal quantity of distilled water were given for 14 days. Three hours were allowed for each

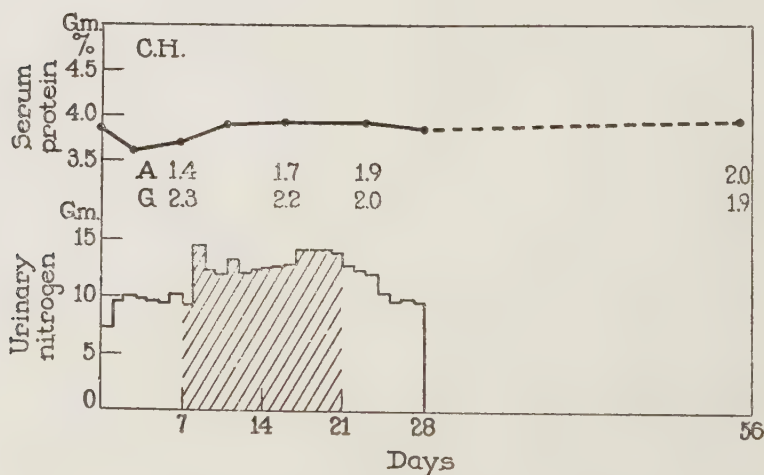


FIG. 1.

The level of serum proteins and urinary nitrogen output are shown before, during and after administration of amino acids (Case I). The urinary nitrogen is shown in blocks representing the amount in grams during one 24-hour period. The cross-hatched area indicates the period during which 500 cc of 5% amino acid solution was injected daily. The figures beneath the graph of the serum proteins represent the amounts of albumin (A) and globulin (G). Change in level of proteins did not occur.

injection. After the series of injections was completed, a small but definite rise in serum proteins took place. The level continued to rise and reached a peak of 5.5% 36 days later. The increase occurred chiefly in the albumin fraction. During the next 3 months, the serum proteins fell slowly to the previous level. Four months later the subject was restudied under the same conditions. On this occasion, larger amounts, 300 cc of 15% solution of amino acids, were given daily for 16 days in 2 injections each lasting 3 hours. Untoward reactions have not been noticed following injections in the amounts used so far. During the first week of administration a perceptible rise in plasma proteins was noticed (Fig. 2). The level continued to rise and a peak of 5.9% was reached 12 days following cessation of therapy. The increase in serum protein again took place in the albumin fraction. An effort is now being made to maintain this level by oral administration.

Both weight curves remained fairly constant and although blood volumes were not measured, it seems unlikely that they changed materially since the hematocrit figures remained relatively constant. Throughout there was no alteration in output of nitrogen derived from urinary albumin. The entire diet was regularly consumed by the first subject but irregularities due mainly to morphine addiction occurred in the second.

*Discussion.* Although it was impossible to carry out total nitrogen balance studies, we have calculated from the approximate nitro-

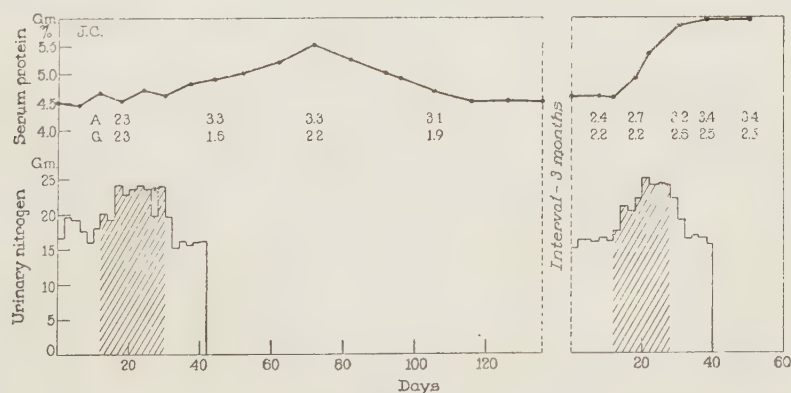


FIG. 2.

The level of serum proteins and urinary nitrogen output are shown before, during and after administration of amino acids (Case II). The urinary nitrogen is in blocks representing the amount in grams during two 24-hour periods. The cross-hatched areas indicate the periods during which amino acid solutions were given. During the first period, 180 cc of a 20% amino acid solution were given; during the second, 300 cc of 15% solution. \*During this 2-day period, administration of amino acids was omitted. Note sharp drop in urinary nitrogen.



gen intake, and from the output of nitrogen in the urine, that probably less than 5% of the amino acids were retained, except perhaps during the first few days of administration. It seems clear that in the second case, the intravenous administration of amino acids initiated regeneration of plasma protein. Evidently sufficient amounts were retained to form new plasma protein. Farr and MacFadyen<sup>5</sup> have shown in careful nitrogen balance studies that nephrotic children are able to utilize intravenously administered amino acids but do not build plasma proteins. According to Whipple's<sup>6</sup> concept of protein storage, it is likely that the amino acids replenish depleted protein stores.

*Conclusion.* In one of 2 hypoproteinemic individuals exhibiting the "nephrotic stage" of chronic nephritis, intravenous administration of amino acids was, on two occasions, followed by a well marked rise in the level of the serum proteins. The rise occurred in the albumin fraction. The other subject failed to respond.

## 13113

**Production of *Cl. welchii* Toxin in Peptone-free Medium.**

JOSEPH T. TAMURA,\* ALFRED A. TYTELL, MILFORD J. BOYD\* AND MILAN A. LOGAN. (Introduced by A. B. Hastings.)

*From the Departments of Bacteriology and Biological Chemistry, College of Medicine, University of Cincinnati.†*

*Clostridium welchii* is customarily cultivated in glucose-peptone beef-infusion broth for toxin production. Many workers have found highly variable yields of toxin, which is usually due to the variation in different lots of peptone. Reed and his coworkers<sup>1</sup> have grown various strains of *Clostridia* in a medium consisting of 5% gelatin and 1% peptone with maximal hemotoxin production by

<sup>5</sup> Farr, Lee E., and MacFadyen, Douglas A., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **42**, 444.

<sup>6</sup> Whipple, George H., *Am. J. Med. Sc.*, 1938, **196**, 609.

\* Two members are indebted to the Craig Yeiser Fund for support during this work.

† This research was supported in part by a grant from the National Research Council, Division of Medical Sciences.

<sup>1</sup> Reed, G. B., Orr, J. H., and Baker, Mary C., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **42**, 620.

*Cl. welchii* ranging from 1:1000 to 1:4000 dilutions which hemolyzed 50% of a 2% suspension of rabbit's red blood cells. Recently Gladstone and Fildes<sup>2</sup> have reported successful cultivation of various *Clostridia* in C.C.Y. media (casein acid-hydrolysate, casein tryptic-digest, yeast extract) without reference to toxin-production.

The writers have undertaken a study of the nutritional requirements and toxin-production of *Cl. welchii* in a simplified medium with the object of producing consistent yields of toxin. The basal medium consists of:

MgSO <sub>4</sub>	0.02 g
Na <sub>2</sub> HPO <sub>4</sub> · 12H <sub>2</sub> O	5.76 "
KH <sub>2</sub> PO <sub>4</sub>	0.24 "
l-tryptophane	0.10 "
Casein acid-hydrolysate 0.28% total N	1000 ml
pH adjusted to 7.9; autoclaved at 10 lb for 10 min.	
After autoclaving, glucosamine hydrochloride and glucose were added to give a final concentration of 0.1% and 0.2% respectively.	

Casein acid-hydrolysate was prepared according to the method employed by Mueller and his coworkers.<sup>3, 4</sup> As expected, the basal medium alone did not support the growth of *Cl. welchii*. The U. S. Public Health strain of *Cl. welchii* SR12 was used throughout the experiments.

Growth was successfully obtained by addition to the medium of 0.1% liver extract.<sup>5</sup> Equally good growth was obtained by substituting pantothenic and pimelic acids for the liver-extract (Table I). Addition of riboflavin and nicotinic acid was found necessary for toxin-production (Table I).

After 5 or 6 passages through pigeons the organisms were cultured in glucose beef-infusion broth for 5 hours or less. Bacterial cells were transferred into a sterile tube, centrifuged and washed with sterile saline twice before inoculation into the medium for toxin production. Unless otherwise stated the cultures were incubated at 37°C for 17 hours. There was sufficient buffer in the medium to prevent a fall in pH to less than 6.1 to 6.2 in 17 to 22 hours' cultivation. Although small amounts of liver-extract (0.01 to 0.05%) gave satisfactory growth, more uniform toxin-production followed the addition of 0.1% liver-extract to the medium.

Toxins have so far been produced in batches of media not ex-

<sup>2</sup> Gladstone, G. P., and Fildes, P., *Brit. J. Exp. Path.*, 1940, **21**, 161.

<sup>3</sup> Mueller, J. H., *J. Immunol.*, 1939, **37**, 103.

<sup>4</sup> Mueller, J. H., and Johnson, E. R., *J. Immunol.*, 1941, **40**, 33.

<sup>5</sup> ‡ Mother-liquor from the alcohol-precipitation of crude liver-extracts. One g of the extract equals 300 g of fresh liver.

ceeding 40 ml in volume. To avoid loss of toxin through adsorption by filtration, the cultures were centrifuged at 4000 rpm for one hour and the supernates carefully pipetted off. The toxins were tested in mice weighing 17 to 22 g, and in pigeons of 275 to 300 g; 50% hemolysis of a 2% suspension of rabbit's red blood-cells in the highest dilution was taken as the titer of hemotoxin.

Since almost all the iron had first been removed from the casein acid-hydrolysate, various concentrations of  $\text{FeSO}_4$  were added to the medium. The optimal concentration for consistent production of toxin was found to be 0.20 to 0.25 mg Fe per liter. A definite amount of iron in the media has previously been found necessary for production of diphtheric toxin by Pappenheimer and Johnson.<sup>5</sup> Variation in the phosphate content of the medium greatly influenced morphology, growth, and toxin-production. When the phosphate content of the final medium was increased from 0.56 to 1.11 mg P/ml the organisms became coccoid in form, and growth and toxin-production decreased.

Quantitative estimates of growth were made by determinations of bacterial nitrogen in twice-washed packed cells from given volumes of culture. These values are recorded in Table I.

TABLE I.  
Representative Growth and Toxin-production of *Cl. welchii* in Peptone-free medium.

Basal medium*	Accessory factors. Liver extract, %	Final pH	Bacterial N, mg/40 ml	Hemotoxin titration 50% hemolysis of 2% rabbit, r.b.c.	M.L.D. for mouse, ml	M.L.D. for pigeon per 100 g, ml
1	.1	6.2		1:20000	.05	
2	.1	6.2	2.3	1:10240	.1	.03
3	.2	6.1	2.4	1:20480	.1	.05
4	.2	6.2	2.3	1:10240	.1	.05
5†	.2	6.4	2.1	1:10240	.1	.06
6†	.1	6.6	2.0	1:10240		.08
7	‡	6.3	2.0	1:640	none	none
8	§	6.2	2.3	1:12800	.1	.08
Peptone beef-infusion broth with 0.25% glucose		5.2 to 5.7	2.9	1:5120	.1	.08

\*Formula is given in the text.

† $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 12.6 g,  $\text{KH}_2\text{PO}_4$ , 0.4 g per liter.

‡Calcium d-pantothenate and pimelic acid, 2μg/ml.

§Calcium d-pantothenate and pimelic acid, 2μg/ml, nicotinic acid, 5μg/ml, riboflavin, 0.2 μg/ml.

<sup>5</sup> Pappenheimer, A. M., Jr., and Johnson, S. J., *Brit. J. Exp. Path.*, 1936, **17**, 335.

*Summary and Conclusions.* *Cl. welchii* was grown in a medium free from peptone or other substances of large molecular weight. Toxins were consistently produced which were equal to or more potent than those produced in glucose peptone beef infusion broth. The pH of the culture media did not fall below 6.0. This may in part be responsible for the higher potency of toxin obtained in this medium. The basal medium alone did not support growth; when it was supplemented with pantothenic acid, pimelic acid, nicotinic acid, riboflavin, or liver-extract, there was a marked increase of growth and toxin-production.

The fact that the highest bacterial-nitrogen value was obtained in glucose-peptone beef-infusion broth, appears to indicate that the casein hydrolysate still lacks one or more chemical ingredients required for maximal growth.

The work is being continued in an effort to identify a chemically defined medium for maximal growth and consistent toxin production by *Cl. welchii*.

The authors are indebted to Merck and Company for generous quantities of the known accessory growth-factors and to Dr. Y. Subbarow of Lederle Laboratories, Inc., for the liver-extract.

### 13114 P

#### Retention of Radiophosphorus in Whole and Aliquot Portions of Tissues of Patient Dead of Leukemia.

L. A. ERF.\* (Introduced by J. H. Lawrence.)

*From the Crocker Radiation Laboratory, University of California, Berkeley.*

This note presents the radioactive phosphorus ( $P^{32}$ ) and the non-radioactive phosphorus ( $P^{31}$ ) content of tissues obtained from a patient dead of chronic lymphoid leukemia, who had received orally 19 days before death a single administration of a sodium phosphate solution, containing radio-phosphorus, which emitted 20 millicuries of beta radiation on the day of administration.<sup>1</sup>

*Materials and Methods.* The radio-phosphorus was produced by the Berkeley cyclotron.<sup>2</sup> The patient was a 65-year-old carpenter

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\* Wm. R. Kenan, Jr., Fellow.

<sup>1</sup> Lawrence, J. H., Scott, K. G., and Tuttle, L. W., *New International Clinics*, 1939, **3**, 33.

<sup>2</sup> Lawrence, E. O., and Cooksey, D., *Phys. Rev.*, 1936, **50**, 1131.



TABLE I.

	Total wet wt. in g.	Whole organs $\mu\text{c/g.}$ wet wt.	Mg of $\text{P}^{31}$ per g. wet wt.	$\mu\text{c/mg}$ $\text{P}^{31}$	Aliquot samples of whole organs $\mu\text{c/g.}$ wet wt.
	(1)	(2)	(3)	(4)	(5)
1. <i>Aorta</i>	122.0	.03	1.25	0.18	.02
2. <i>Bladder</i>					
2. Gall bladder	14.2	.02	0.39	.05	.03
3. Urinary bladder	86.5	.02	0.38	.04	.02
4. <i>Bone</i>					
4. Calverium	55.5	.02	87.1	.0002	.01
5. Femur diaphysis	192.5	.04	92.8	.0004	.01
6. Femur epiphysis	441.9	.04	44.4	.0007	.02
7. Ribs (12)	142.0	.09	46.1	.0016	.16
8. Tibial diaphysis	123.9	.03	89.5	.0003	.02
9. Sternum	11.0	.08	19.9	.0033	.12
10. Vertebral bodies (thoracic and lumbar)	84.3	.09	20.8	.0038	.08
11. <i>Brain</i>					
11. Cerebellum	175.0	.03	2.72	.0093	.03
12. Cerebrum	1192.0	.03	1.97	.013	.02
13. <i>Colon</i>	393.0	.03	1.46	.016	.03
14. <i>Cord</i> , Spinal	18.6	.03	2.18	.01	.03
15. <i>Duodenum</i>	120.5	.05	1.46	.028	.04
16. <i>Fat</i> , mesenteric	61.0	.02	0.30	.041	.01
17. <i>Eyes</i>	17.0	.02	0.51	.031	
18. <i>Ileum</i>	211.0	.03	0.81	.032	.03
19. <i>Jejunum</i>	311.0	.03	1.24	.022	.04
20. <i>Kidney</i>	278.5	.06	1.22	.045	.05
21. <i>Liver</i>	1830.0	.09	1.57	.047	.07
22. <i>Lung</i>	1305.5	.04	1.20	.03	.04
23. <i>Lymph nodes</i>					
23. Bronchial	15.8	.08	1.64	.039	.06
24. Peripheral	34.5	.07	3.08	.019	.06
25. <i>Marrow</i>					
25. Tibia, diaphyseal marrow	20.5	.03	11.48	.0025	.07
26. Femur, epiphyseal "	63.6	.04	26.6	.0012	.03
27. Femur, diaphyseal "	37.9	.03	11.29	.0026	.03
28. <i>Muscle</i>					
28. Diaphragm	159.0	.03	1.25	.021	.03
29. Heart	403.0	.04	1.16	.029	.05
30. Rectus femoris	300.5	.05	1.77	.022	.04
31. Tongue	74.0	.06	1.35	.036	.05
32. <i>Pancreas</i>	113.5	.02	1.22	.015	.03
33. <i>Prostate</i>	16.3	.04	3.21	.011	.04
34. <i>Skin</i>	228.0	.01	0.82	.016	.01
35. <i>Spleen</i>	910.0	.07	1.60	.035	.04
36. <i>Stomach</i>	193.0	.04	1.08	.032	.04
37. <i>Testes</i>	16.8	.06	1.67	.031	.05
38. <i>Thyroid</i>	10.3	.04	1.83	.017	.04

who on August 16, 1940, complained of fatigue and headaches of 2 weeks' duration, and who on physical examination had an enlarged spleen and enlarged inguinal glands. The blood findings were: Hemoglobin, 37%; r.b.c., 2,000,000; w.b.c., 125,600. 99% of the latter were small leukemic lymphocytes. On October 20, 1940, the

patient took orally 120 cc of a solution containing 3.6 g of sodium phosphate and emitting 20 millicuries of beta radiation. Between 10/28/40 and 11/7/40 the patient received 2350 cc of blood without marked alterations in the blood findings. The patient died 11/8/40. Fortunately it was possible to obtain at postmortem all of the organs and one entire lower extremity. Typical gross pathological findings of leukemia were observed. Thirty-eight organs and tissues were weighed as were small aliquots (5 g approx.) of each. Then the whole organs and tissues and their aliquots were placed in separate crucibles and all ashed at 400°C. The ashes (all of which weighed less than 200 mg each) of the aliquots were immediately measured for radioactivity by use of an electrometer. The ashes of the whole organs and tissues were thoroughly mixed and 100 mg aliquots were assayed for radioactivity. Quantitative phosphorus analyses were made by the method of Pregl.<sup>3</sup>

*Results.* The microcuries per gram wet weight of the whole organs and tissues (Column 2) and their respective aliquots (Column 5) and the total number of milligrams of  $P^{31}$  per gram wet weight (Column 3) of the samples are listed in Table I. In 13 (Nos. 2, 3, 8, 13, 14, 15, 16, 18, 28, 32, 34, 36, and 38) the concentrations of  $P^{32}$  were quite similar in the ash of the aliquot and in the ash of the respective whole organ. In 7 (Nos. 4, 5, 7, 9, 19, 25, and 29) the concentration of  $P^{32}$  was greater in the ash of the aliquot while the opposite was true in the remaining 17 samples. Aliquot samples of the eyes were not taken. In Column 4 is listed the "specific radioactivity," or the microcuries per mg of  $P^{31}$  of each tissue.

*Discussion.* The variations of the  $P^{32}$  content of the ash of the whole organs and their respective aliquots is probably due to the fact that small aliquots of organs cannot be representative samples as most organs are not uniform throughout in structure or function.

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<sup>3</sup> Pregl, F., and Roth, H., *Quantitative Organische Analyse*, Julius Springer, Berlin, 1935.

## Androgen and Spermatogenesis in the Hypophysectomized Guinea Pig.\*

EUGENE CUTULY.

*From Wayne University College of Medicine, Detroit, Mich.*

In a number of species spermatogenesis has been maintained after hypophysectomy by androgens (the rat,<sup>1-4</sup> the mouse,<sup>5</sup> and the rabbit<sup>6</sup>). The only report on guinea pigs is that of Scowen,<sup>7</sup> who failed to maintain spermatogenesis with androsterone and testosterone in animals hypophysectomized for 20 days. The data which follow are not in agreement with those of Scowen, but show that the tubular tissue of the testis in the hypophysectomized guinea pig responds to androgen as does that of the rat, mouse and rabbit.

It should be stated that experiments on spermatogenic maintenance or on reestablishment of spermatogenesis in the adult guinea pig must be continued for long periods to be valid. This is due to the fact that atrophy of the germinal epithelium after ablation of the pituitary is not rapid in this species. Allanson, *et al.*,<sup>8</sup> found sperm in testes of guinea pigs hypophysectomized for as long as 56 days. Observations on 2 untreated animals (Table I, Nos. 5 and 6) show that sperm may persist for as long as 66 and 84 days after removal of the hypophysis. Serial sections of the pituitary capsule were made to check completeness of the hypophysectomy.

Four adult guinea pigs (Nos. 1, 2, 3, and 4) were hypophysectomized for periods ranging from 60 to 155 days. Daily subcutaneous injections of 3 mg of testosterone propionate\* were begun

\* This study was aided in part by Works Project Administration No. 665-51-3-225.

<sup>1</sup> Walsh, E. L., Cuyler, W. K., and McCullagh, D. R., *Am. J. Physiol.*, 1934, **107**, 508.

<sup>2</sup> Nelson, W. O., and Gallagher, T. F., *Science*, 1936, **84**, 230.

<sup>3</sup> Cutuly, E., McCullagh, D. R., and Cutuly, E. C., *Am. J. Physiol.*, 1937, **119**, 121.

<sup>4</sup> Nelson, W. O., and Merckel, C. G., *PROC. SOC. EXP. BIOL. AND MED.*, 1937, **36**, 825.

<sup>5</sup> Nelson, W. O., and Merckel, C. G., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **38**, 737.

<sup>6</sup> Greep, R. O., *Anat. Rec.*, 1939, **73** (suppl.), 23.

<sup>7</sup> Scowen, E. F., *Anat. Rec.*, 1938, **70** (suppl.), 71.

<sup>8</sup> Allanson, M., Hill, R. T., and McPhail, M. K., *J. Exp. Biol.*, 1935, **12**, 348.

\* Testosterone propionate was supplied by Dr. Erwin Schwenk, Schering Corporation, Bloomfield, N.J.

TABLE I.  
Effect of 3 mg Daily of Testosterone Propionate on the Hypophysectomized Male Guinea Pig.

No. of animal	Initial body wt, g	Body wt at autopsy, g	No. of days hypophysectomized	Autopsy wt		% loss in adrenal wt from normal avg
				Testes, g	Adrenals, g	
1	478	444	62	2.16	.043	88
2	500	616	60	1.12	.102	72
3	630	1010	127	2.76	.087	76
4	568	920	155	2.76	.064	83
5	560	780	66	1.69	.244	33
6	697	770	Hypophysectomized control 84	1.24	.162	56
7	440	730	Hypophysectomized control Sham-operated control	3.57	.327	
8	—	805	Normal control	4.15	.418	
9	—	680	" "	3.30	.357	
		Avg normal control wt		3.67	.367	

the day after hypophysectomy and continued until the day preceding autopsy. In each instance the testes weighed less than those of normal controls. However, except in animal No. 2 where germ cell preservation was not so thorough, histological differences in the tubules of treated and normal animals were not easily discernible. In the testes of the androgen-treated hypophysectomized animals the majority of tubules exhibited active spermatogenesis at autopsy, although a few were profoundly atrophied. In the untreated hypophysectomized control animals, on the other hand, many tubules were markedly atrophic, some were degenerating, while others had an epithelium showing cells in all stages of gametogenesis, including spermatozoa. Testes of treated and untreated hypophysectomized pigs alike differed from normal testes in that interstitial cells were atrophied. At autopsy vast numbers of motile sperm were found in the epididymides of hypophysectomized guinea pigs injected with testosterone propionate, and considerable numbers were present in the epididymides of uninjected hypophysectomized animals.

Two additional facts may be ascertained from the data in Table I. First, there was shrinkage of the adrenal glands in treated and untreated hypophysectomized animals. The autopsy weights of the adrenals of these animals showed reductions ranging from 33 to 88% below the normal control average. Second, the adult hypophysectomized guinea pig, unlike the adult hypophysectomized rat, may gain considerably in body weight. This finding was unexpected, although it corroborates a similar observation made by Schweizer,<sup>9</sup>

<sup>9</sup> Schweizer, M., Charipper, H. A., and Kleinberg, W., *Endocrinology*, 1940, **26**, 979.



*et al.*, who noted a weight increment in several hypophysectomized male pigs with unsuccessful ocular pituitary grafts.<sup>†</sup> Moreover, examination of the data published by Allanson, *et al.*,<sup>8</sup> indicates a post-operative weight gain in 3 of their hypophysectomized males, although no special mention was made of the fact. A possible explanation of the gain in body weight after hypophysectomy may lie in the fact that, shortly after the guinea pig has recovered from effects of the operation, it eats as voraciously as the normal animal. The rat, on the contrary, has a diminished food intake after hypophysectomy. Thus it would appear that appreciable gain in weight in the guinea pig, at least, is not necessarily dependent on a growth factor from the pituitary.

*Summary.* Spermatogenesis was maintained by subcutaneous injection of 3 mg daily of testosterone propionate in 4 guinea pigs hypophysectomized for 60 to 155 days. Tubular atrophy in the pig may not be complete as late as 84 days after hypophyseal ablation. Adrenal atrophy occurs after removal of the pituitary, but increase in body weight does not necessarily cease.

### 13116 P

#### Acetylation of Optical Isomers of S-benzylcysteine in Rats and Humans.

JAKOB A. STEKOL.

*From the Department of Chemistry, Fordham University, New York City.*

We reported the conversion of S-benzyl-*l*-cysteine to the corresponding acetyl derivative in the rat, rabbit, dog<sup>1</sup> and man<sup>2</sup> and suggested the possibility of the inversion of S-benzyl-*d*-cysteine to the acetylated *l*-derivative *via* Knoop's acetylation mechanism<sup>3</sup> which postulates the formation of a keto acid from the *d*-amino acid followed by the interaction of the keto acid with ammonia and pyruvic acid to give the *l*-acetyl amino acid. duVigneaud, *et al.*,<sup>4</sup> reported

<sup>†</sup> Gains in body weight have been observed to occur also in hypophysectomized female guinea pigs with unsuccessful ocular grafts of pituitary (Haterius, H. O., and Cutuly, E., unpublished data).

<sup>1</sup> Stekol, J. A., *J. Biol. Chem.*, 1938, **124**, 129; 1939, **128**, 199.

<sup>2</sup> Stekol, J. A., *Proc. Am. Chem. Soc.*, 100th meeting, Detroit, Mich., 1940.

<sup>3</sup> Knoop, F., *Z. physiol. Chem.*, 1910, **67**, 489; Knoop, F., and Kertess, E., *Z. physiol. Chem.*, 1911, **71**, 252; Knoop, F., and Blanco, J. G., *Z. physiol. Chem.*, 1925, **146**, 267.

<sup>4</sup> du Vigneaud, V., Wood, J. L., and Irish, O. J., *J. Biol. Chem.*, 1939, **129**, 171.

results which apparently indicated such an inversion of S-benzyl-*d*-cysteine in the rat. On feeding the *d*-isomer to rats, some *l*-acetyl derivative together with *dl*-acetyl benzylcysteine were isolated from the urine. When *d*-benzylcysteine was fed to rats and humans,<sup>2</sup> significantly smaller yields of the acetylated products could be isolated from the urine as compared to those obtained after the administration of comparable amounts of *l*-benzylcysteine. We drew the conclusion at the time<sup>2</sup> that these findings did not conform to the postulates of Knoop's acetylation theory which presupposes that if the acetylation of an amino acid takes place *via* the keto acid, then the amounts of the acetylated product excreted in the urine of animals which were fed *d*- or *l*- amino acid should be the same. Indeed, du Vigneaud and Irish,<sup>5</sup> who revived Knoop's theory, stated that "this would be expected if the theory were correct, since both enantiomorphs should be deaminated to the keto acid." The lower yields of the acetyl derivatives in the urine after the feeding of *d*-benzylcysteine to rats and man<sup>2</sup> we later attributed<sup>6</sup> to a possible destruction of the intermediate keto acid which was presumably formed from *d*-benzylcysteine prior to its conversion to the acetyl derivative.

Before accepting the acetylation mechanism of Knoop's as valid for the synthesis of mercapturic acids *in vivo*, we felt it advisable to ascertain experimentally the fate of the larger portion of *d*-benzylcysteine when it is fed to animals, since so far only a fraction of the substance fed has been accounted for in the urine as the acetylated product. We, therefore, repeated our earlier work on the isomers of benzylcysteine in rats and humans<sup>2</sup> and isolated from the urine the acetylated derivatives as before. In addition, however, we were able to isolate from the urine of rats and humans which were fed *d*- or *dl*-benzylcysteine, *unacetylated d-benzylcysteine* in 20 to 28% yields of the *d*-derivative fed. At no time comparable amounts of *l*-benzylcysteine yielded in the urine the unchanged substance, but only the *l*-acetyl derivative. Control experiments excluded the possibility of hydrolysis or racemization of the acetyl derivative during the isolations. The isolated *d*-benzylcysteine had a specific rotation of  $(\alpha)_D^{24} = -24^\circ$  for a 1% solution in 1 N NaOH. Analytically, the substance was identical with the original *d*-benzylcysteine fed.

This observation suggests that if both isomers of benzylcysteine are acetylated *in vivo* directly (which is the more likely probability) then the *l*-isomer is acetylated *in vivo* to a greater extent than the

<sup>5</sup> du Vigneaud, V., and Irish, O. J., *J. Biol. Chem.*, 1937-38, **122**, 349.

<sup>6</sup> Stekol, J. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **45**, 693.

*d*-enantiomorph. If the acetylation is preceded by the formation of the corresponding keto acid from both isomers, as Knoop's theory postulates, then the *d*-isomer is apparently less readily deaminized than the *l*-isomer. *d*-Benzylcysteine is undoubtedly directly acetylated, as is indicated by the excretion of *dl*-acetylbenzylcysteine in the urine. We are not excluding the possibility of racemization of *d*-benzylcysteine *in vivo*, neither do we imply that some oxidative deamination of either or both isomers of benzylcysteine does not occur *in vivo*.

The work is being continued and extended to benzylhomocysteine and phenylaminobutyric acid, particularly with reference to the possibility of racemization and preferential rate of acetylation of the optical isomers of these amino acid derivatives *in vivo* as contrasted with Knoop's acetylation theory.

### 13117

#### Some Effects of Experimental Trichinosis in the Dog.\*

EDGAR H. BEAHM AND MYRON N. JORGENSEN. (Introduced by F. C. Hill.)

*From the Department of Bacteriology and the Department of Biochemistry, Creighton University School of Medicine, Omaha, Nebraska.*

In view of the fact that encysted trichina larvae become calcified in the muscles of the host, it appeared desirable to determine the changes which occur in the blood picture of dogs suffering from experimental trichinosis. The present report presents the results of cytological studies in which concurrent determinations were made on the level of blood calcium, hemoglobin and inorganic phosphate at periodic intervals before and during the period of infection.

*Methods and Technic.* The 13 mature mongrel dogs, used in these experiments, were kept under observation for several weeks to determine whether or not they were suffering from any disorder. Each was treated with arecoline hydrobromide (Parke, Davis) to insure freedom of the gastro-intestinal tract of parasites. The dogs were kept in separate cages in a room in which the temperature fluctuated very little. Purina dog biscuit was supplied *ad libitum*.

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\* The authors wish to acknowledge the assistance of John E. Krettek with the blood counts.

TABLE I.  
Blood Picture in Trichina infected Dogs.

Sex and Number	Wt in kg	Control period in days	Control period			Experimental period			
			R.B.C. in millions per mm <sup>3</sup>	Hematocrit	Grams % Hemoglobin	Organisms fed (in thousands)	R.B.C. in millions per mm <sup>3</sup>	Hematocrit	Grams % Hemoglobin
M3	6.81	13	5.80-7.25	44.49	14.9-16.9	90	5.30-6.70	40.44	12.4-16.7
M2	11.81	20	6.90-7.25	46.48	14.7-17.7	160	6.45-7.00	44.48	15.3-18.1
M1	15.90	20	6.85-7.45	47.48	14.2-18.0	220	5.85-7.00	42.56	13.9-16.4
M4	8.63	13	6.50-8.90	55.66	16.3-19.4	292	5.48-7.56	44.55	13.5-16.7
M9	7.50	13	6.20-6.50	41.42	11.4-12.6	300	5.33-6.74	42.45	12.6-13.4
F5	7.27	8	6.00-7.10	44.49	15.0-15.2	400	5.07-6.12	39.46	9.5-12.6
M7	7.27	22	5.07-5.44	41.47	10.5-11.8	480	4.19-5.22	32.42	10.4-12.6

TABLE II.  
Leucocyte Variations in Trichina-infected Dogs.

Sex and Number	Control period			Experimental period				After recovery*	
	W.B.C. in thousands	Eosinophils %	Days after infection	W.B.C. in thousands	Eosinophils %	W.B.C. in thousands	Eosinophils %	W.B.C. in thousands	Eosinophils %
M3	10.1-18.0	1-3	11-17	16.7-11.5	14-11	12.7	12.7	12.7	2
M2	14.0-15.0	0-5	10-23	14.7-14.5	21-10	15.2	15.2	15.2	7
M1	15.2-16.0	0-3	10-14	20.3-18.1	11-17	14.6	14.6	14.6	2
M4	9.3-15.0	2-10	6-22	12.0-13.0	21-24	10.9	10.9	10.9	9
M9	12.4-17.6	2-4	15-30	20.0-17.0	18-8	14.8	14.8	14.8	14
F5	9.5-12.5	4-6	10-17	19.2-10.8	38-15	15.0	15.0	15.0	3
M7	11.1-12.8	0-8	14-22	10.6-14.2	25-8	10.1	10.1	10.1	5
M10	14.1-17.3	2-7	12-26	14.9-13.0	19-8	12.3	12.3	12.3	7
M12	11.7-16.0	0-3	12-18	12.2-15.3	22-4	12.9	12.9	12.9	4
F13	15.5-18.8	0-2	12-20	16.0-14.0	16-6	13.2	13.2	13.2	4

\* After 30 days.



Blood was drawn from the jugular vein using 30 mg of potassium oxalate as an anticoagulant for each 5 cc sample. Samples were taken in the forenoon on animals which were fasted 16 hours.

Total counts were made with a bright-line Neubauer hemacytometer. Blood smears were made on a coverslip and stained with Wright's stain. Two hundred to 400 cells were tabulated, alternately, in the differential counts. Hematocrit determinations were made with a Wintrobe tube using 1.1 as the shrinkage correction factor.

Serum calcium was determined by the Clark-Collip<sup>1</sup> modification of the Tisdall method. Hemoglobin and inorganic phosphate were determined according to the method of Van Slyke<sup>2</sup> and Kuttner-Lichtenstein<sup>3</sup> respectively. All analyses were made in duplicate.

The dogs were infected by feeding them 90 to 480 thousand trichina larvae contained in fresh rabbit muscle. Three of them were discarded during the period of infection due to poor physical condition. All animals were shown to be infected at autopsy.

*Results.* The normal blood picture of each dog was followed for a period of 13 to 22 days since it appeared desirable that emphasis should be placed upon changes observed rather than on absolute values. Table I shows the results of determinations conducted on these animals for a period of 60 days. Normal erythrocyte values and differential counts fall within the range of normal values.<sup>4, 5, 6</sup>

TABLE III.  
Blood Picture in Trichina-infected Dogs.

Sex and Number	Control period			Experimental period		
	Serum Calcium in mg per 100 cc	Eosinophils, %	Inorganic phosphate, mg%	Serum calcium, mg%	Eosinophils, %	Inorganic phosphate, mg%
M3	11.06-12.68	1-3	5.58-5.88	14.50-10.40	14-11	4.01-7.20
M2	10.50-11.85	0-5	4.40-5.30	12.50- 9.60	21-10	4.06-7.30
M1	9.50-11.60	0-3	4.50-6.00	13.00-10.60	11-7	3.69-5.20
M4	10.60-13.20	2-10	3.90-4.80	16.50-11.00	21-24	3.30-3.80
M9	10.30-10.90	2-4	5.75-5.88	18.70-12.00	18-8	5.30-7.40
F5	12.30-13.80	4-5	4.85-7.20	12.60-10.50	38-15	4.40-6.98
M7	11.06-11.88	0-8	4.58-5.13	16.13-11.10	25-8	4.04-5.65
M10	9.81-11.17	2-7		9.76-10.52	19-8	
M12	10.44-10.96	0-3		11.28-10.83	22-4	
F13	10.60-11.14	0-2		10.49-10.36	16-6	

<sup>1</sup> Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, **63**, 461.

<sup>2</sup> Hawk and Bergeim, *Practical Physiological Chemistry*, 11 Ed., 533.

<sup>3</sup> Kuttner, T., and Lichtenstein, L., *J. Biol. Chem.*, 1930, **86**, 671.

<sup>4</sup> Scarborough, R. A., *Yale J. Biol. and Med.*, 1930, **3**, 359.

<sup>5</sup> Wintrobe, M. M., Shumaker, H. B., and Schmidt, W. J., *Am. J. Physiol.*, 1936, **114**, 502.

<sup>6</sup> Morris, M. L., Stelton, N. J., Allison, J. B., and Green, D. F., *J. Lab. and Clin. Med.*, 1940, **25**, 353.

White blood counts were slightly higher (Table II) than those found by Morris, *et al.*<sup>6</sup> The variations observed in serum calcium ranged from 9.8 to 13.8 mg %, average 11.3. Inorganic phosphate ranged from 3.9 to 6 mg % with an average of 4.87.

During the period of infection the cell volume, total erythrocyte count and hemoglobin remained essentially unaltered (Table I). The white cell counts, both total and differential, showed striking variations from the normal in all animals. The leucocyte count (Table II) showed an increase in those animals having the most severe infection. Even those dogs receiving 13 to 14 thousand trichina larvae per kilogram body weight showed marked changes. The most marked variation in the blood picture was in the differential count. There was a gradual increase in the eosinophiles (Column 6, Table II) which reached a maximum (11 to 38%) 10 to 15 days after infection. No significant changes were observed in other leucocytes which is in contrast with the findings of Beahm and Downs<sup>7</sup> in the albino rat.

Significant increases in the serum calcium level (Table III) were

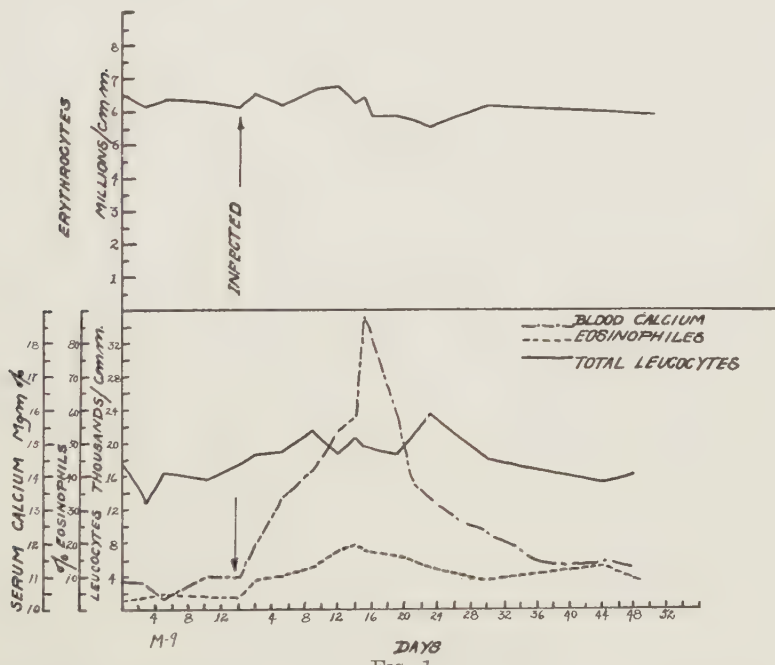


FIG. 1.

Graph showing comparison of results from control and experimental period of trichina-infected dog M9.

<sup>7</sup> Beahm, E. H., and Downs, C. M., *J. Parasitol.*, 1939, **25**, 405.

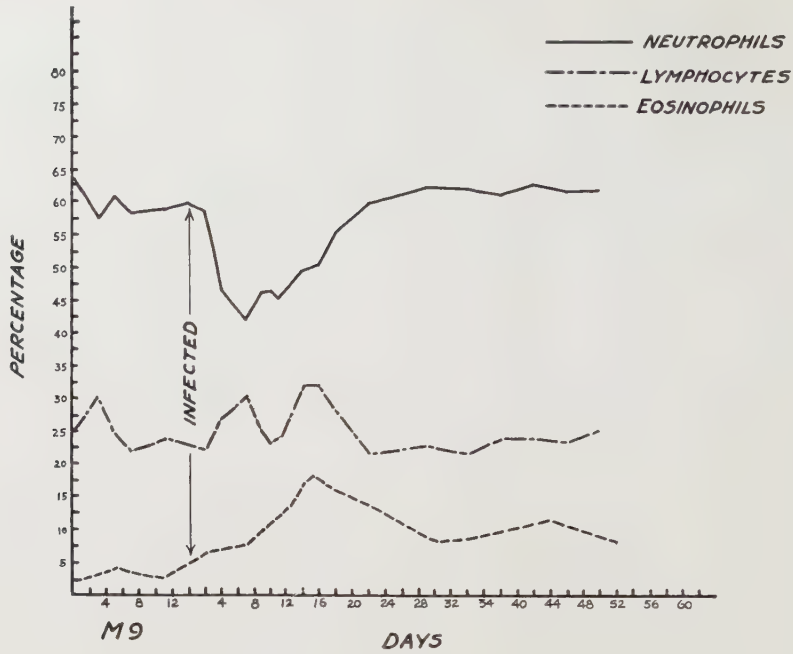


FIG. 2.

Differential count of trichina-infected dog M9.

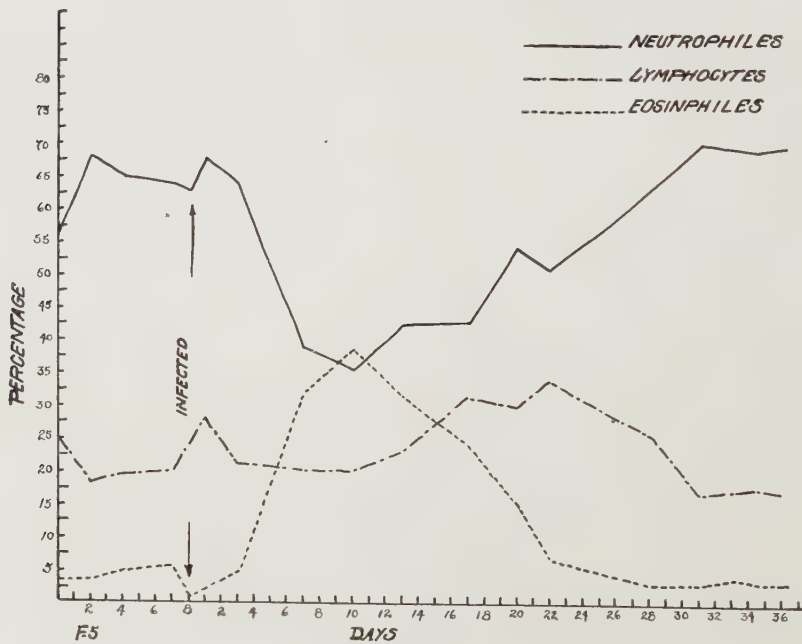


FIG. 3.

Differential counts. Graph showing comparative data obtained during control and experimental period on dog F5.

observed in only 4 dogs suffering from severe trichinosis. These animals (M12, M4, M9, M7) that received 32, 33, 40 and 66 thousand trichina larvae per kilogram of body weight exhibited increases in serum calcium, eosinophiles and total leucocytes. At the same time a moderate decrease was exhibited in the level of inorganic phosphate. Figs. 1 and 2 depict these changes as observed in M9. In contrast with these findings, M10, F13, and F5 had marked eosinophilia but no rise in calcium was observed although they received 34, 40, and 55 thousand larvae per kilogram of body weight. Fig. 3 graphically represents findings observed in F5 which are typical of these animals.

*Summary.* No significant changes in cell volume, total erythrocyte count and hemoglobin values were observed in trichina-infected dogs. All animals exhibited increases in eosinophiles which reached a maximum 10 to 15 days after infection. Significant increases in serum calcium, which reached a maximum (16-18 mg %) 11 to 18 days after infection, were observed in only 4 dogs. It would appear that hypercalcemia can only be elicited by a massive infection, whereas an eosinophilia will always result from ingestion of fewer organisms.

## 13118

**Absorption of Drugs Through the Bone Marrow.**

DAVID I. MACHT.

*From the Pharmacological Research Laboratory, Hynson, Westcott & Dunning, Inc., Baltimore, Md.*

For years the writer has been studying the penetration of drugs into the body through unusual channels and has published papers on their absorption through the bladder, urethra, ureters, vagina, eyes, ears, nose, pharynx, esophagus and pathological versus normal skin and mucous membranes.<sup>1, 2</sup> Having observed that drugs penetrate the tooth pulp and canaliculae,<sup>3</sup> the author determined to investigate what drugs and poisons in general can be absorbed through bone marrow and how rapidly. Such a research was deemed especially desirable because of the recent work of Morrison and Samwick<sup>4</sup>

1 Macht, D. I., *J. A. M. A.*, 1938, **110**, 409.

2 Macht, D. I., *Arch. internat. de Pharmacodyn. et de Therap.*, 1938, **58**, 221.

3 Macht, D. I., *J. Pharmacol. and Exp. Therap.*, 1923, **22**, 123.

4 Morrison, M., and Samwick, A. A., *J. A. M. A.*, 1940, **115**, 1708.



on successful intramedullar transfusion of human bone marrow and the brilliant experiments of Tocantins<sup>5</sup> and O'Neill,<sup>6</sup> who reported efficient absorption of blood, plasma, glucose, congo red and metallic mercury injected into the intramedullar canal of higher animals.

Ten experiments were made on dogs, 30 on cats, 75 on rabbits, 60 on guinea pigs, 20 on rats and 12 on mice. Many drugs and pharmacological agents have been studied and still others are being examined by injection into the intramedullar cavity, injections being generally made into tibia but sometimes into humerus and femur. In rabbits and smaller animals the bones are thin enough to admit of the puncture or boring of a stout needle, the caliber of which must conform to their size. In larger animals trocars of various sizes were used to make small apertures through the bone into the medullar cavity and through these openings metal canulae reached the marrow. Two groups of drugs were employed in this study, *i. e.*, (1) aqueous solutions of potent principles and (2) oils and other liquid drugs.

*Aqueous Solutions.* Well-known potent drugs soluble in water or physiological saline were injected into medullar cavities of various animals and their subsequent absorption was shown by characteristic systemic effects. As indicated by pharmacological responses elicited, all the drugs in the subjoined list were promptly absorbed through the bone marrow.

Morphine sulphate	Effect on respiration
Apomorphine	Emetic action
Cobra venom	Effect on circulation and respiration
Thebaine HCl	Convulsant action
Strychnine nitrate	Convulsions
Metrazol	"
Picrotoxin	"
Caffeine	Effect on circulation and respiration
Ouabain	Effect on heart
Sodium nitrite	Fall in blood pressure, methemoglobin formation
Atropine sulphate	Mydriasis, paralysis of intestines
Pilocarpine HCl	Myosis, salivation, intestinal peristalsis
Physostigmin	Intestinal paralysis, twitching of skeletal muscle, slowing of heart
Nicotine	Typical picture of poisoning, death from paralysis of respiration
Epinephrine	Effect on blood pressure
Ephedrine	" " " "
Aconitine	Typical effect on respiration and circulation
Phenolsulphonphthalein	Excretion by kidneys
Insulin	Convulsions

<sup>5</sup> Tocantins, L. M., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 292.

<sup>6</sup> Tocantins, L. M., and O'Neill, J. F., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 782.

The following protocols illustrate some of the results obtained:

<i>Experiment of March 24, 1941</i>	<i>Experiment of April 4, 1941</i>
Rabbit weighing 2 kg	Rabbit weighing 1.2 kg
Injected pilocarpine HCl, 2 mg to the ce, in tibia	Injected 1 cc of phenolsulphonphthalein in tibia
In 10 min. profuse salivation and mark- edly slowed heart-beat	Output first hour 35%
Myosis, active stimulation of intestinal peristalsis	" second " 50%
Gradual recovery	Total 85%

*Absorption of Oils and Other Liquid Drugs.* The effects of various oils and "oily" solutions injected into the medullary cavity of bones were even more interesting. Thus evipal, introduced into the tibiae of guinea pigs, completely anesthetized them in a few minutes. So did avertin and chlor-butanol dissolved in olive oil. Pure benzyl benzoate, injected into tibia of a cat under paraldehyde anesthesia, was promptly absorbed as indicated by fall in blood pressure and depressed respiration.

Between the volatile or essential oils and the fixed oils there was a marked difference in rapidity of absorption. The volatile oils studied were oils of rose, rose geranium, rosemary, thyme, anise, orange, sassafras, cinnamon, cloves, tansy and wintergreen. Systemic effects from all these were usually noted a few minutes after their introduction into the bone marrow, *i. e.*, primary excitation, followed by depression, convulsions, coma and death, depending on dose injected.

Absorption of the so-called fixed or heavy oils through bone marrow usually proceeded more slowly. Thus chlor-butanol in olive oil, injected into tibiae of cats and guinea pigs, effected general anesthesia in from 5 to 10 minutes. Camphorated oil (N.F.) dissolved in linseed oil produced typical epileptic convulsions in 15 to 30 minutes. A-estradiol in olive oil, injected into tibiae of spayed rats, produced positive oestrus smears after a lapse of 3 days. Crystalline progesterone in sesame oil (Proluton) injected into Clauberg rabbits, effected typical progestational proliferation of the endometrium. Suspensions of heparin in olive oil or peanut oil exerted anticoagulant effects in rabbits for longer periods than intravenous injections of the aqueous solutions. Introduction of a few drops of croton oil into tibiae of guinea pigs and rabbits was followed in 15 to 30 minutes by powerful contractions of abdominal muscles, purgation, ejaculation in males, coma and death (intramuscular injections of this oil had little effect). Castor oil had no laxative effect, thus confirming the view generally held regarding its mechanism of action. Injections of Ruvettus<sup>7</sup> oil, however, were mildly laxative. Doses of

<sup>7</sup> Macht, D. I., and Barba-Gose, J., *J. Am. Pharm. Assn.*, 1931, **20**, 558.

0.2 cc of olive, mineral, peanut, peach kernel, sesame and cottonseed oils, respectively, exhibited no harmful effect in guinea pigs.

*Absorption of Epinephrine.* Introduction of *aqueous* solutions of epinephrine into medullar cavities of bones was promptly followed by a sharp rise in blood pressure with transient depression of respiration and a rapid return of the blood pressure in cats, dogs and rabbits to the normal or frequently below the normal level. This effect differed but little from the response elicited by intravenous injections of the drug in the same animals. When solutions of epinephrine *in oil* were used, however, a very different picture was obtained. Such a solution of epinephrine in peanut oil, injected into the *muscles* of cats, dogs and rabbits, effected no noticeable rise in blood pressure. When the oily solution was injected into a *medullar cavity*, however, a striking pharmacological picture was observed. Such intramedullar injections were followed by a prompt rise in blood pressure to a moderate height, not as marked as that following aqueous solutions of the drug but of long duration, lasting from 20 to 30 minutes (Fig. 1).

*Summary.* (1) The absorption of many drugs introduced into the bone marrow was studied in various animals. (2) Aqueous solutions of all such agents were rapidly absorbed as indicated by their characteristic systemic effects. (3) Intramedullar injections offer an especially useful means for pharmacological study of oils and oily solutions. (4) Volatile oils are rapidly absorbed through bone marrow but the fixed oils are absorbed more slowly, thus retarding absorption of active principles dissolved therein. (5) Epinephrine in oil, introduced into medullary canals, produces a marked and long-sustained rise in blood pressure, an effect which cannot be achieved by intramuscular injection of such an oil solution.

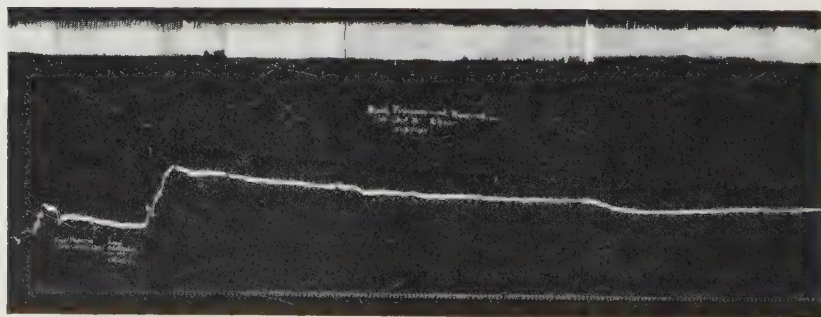


FIG. 1.  
Effect of 2 mg of adrenalin in oil in tibia of cat.

## 13119 P

**Combined Bacteriostatic Activity of Sulfanilamide and Azochloramid upon Group A Hemolytic Streptococcus and Enterococcus.**

ERWIN NETER.

*From the Bacteriological Laboratories, Children's Hospital, and the Department of Pathology and Bacteriology, University of Buffalo, School of Medicine, Buffalo, N.Y.*

The therapeutic effectiveness of sulfanilamide may be markedly enhanced in the presence of other antibacterial agents, notably immune serums. The question presents itself as to whether or not a similar synergistic action may be obtained by the combined use of two different chemotherapeutic substances. *In vitro* studies on the combined bacteriostatic activity upon pneumococci of sulfapyridine or sulfathiazole and optochin hydrochloride (ethylhydrocupreine hydrochloride) or beta hydroxyethylapocupreine dihydrochloride (Parke, Davis & Co.) failed to reveal such a synergistic effect.<sup>1</sup> On the other hand, it could be shown that with *E. coli* as test organism, the combined use of pyridium (phenylazo-alpha-alpha-diaminopyridine mono-hydrochloride) and sulfamido compounds resulted in bacteriostatic effects beyond that exerted by either compound alone.<sup>2</sup> The activity of sulfamido compounds and azochloramid used in combination against hemolytic streptococcus Group A and enterococcus is reported in the following communication. Azochloramid (N, N'-Dichloroazodicarboxamidine) (Wallace and Tiernan products) was chosen as a representative of chlorine compounds. It is a bacteriostatic and bactericidal agent which is relatively stable and whose effectiveness in the presence of organic matter is inhibited to a slight degree only.

As test microorganisms a strain of beta hemolytic streptococcus (Group A Lancefield) which was isolated from the chest fluid of a patient with empyema and a strain of hemolytic enterococcus that was isolated from the urine of a patient with infection of the urinary tract, were used. Brain heart infusion and ¼% maltose phenol red broth (Difco) were employed as culture media. Sulfanilamide [p-aminobenzenesulfonamide, prontosil, repurified for injection (Winthrop)] and sulfathiazole [2-sulfanilamidothiazole (Squibb)] were dissolved in appropriate amounts in broth by heating in a

<sup>1</sup> Neter, E., *J. Bact.*, 1941, **41**, 273.

<sup>2</sup> Neter, E., and Loomis, T. A., *The Urol. and Cutan. Review*, 1941, **45**, 295.



water bath. These solutions as well as the broth media were sterilized by autoclaving at 15 lb pressure for 12 minutes. Azochloramid was dissolved in sterile broth and kept in the dark; the azochloramid-broths were not heated. Controls for sterility were carried out.

The number of viable organisms was determined by means of poured blood agar plates. The specimens were diluted in broth containing p-aminobenzoic acid and/or sodium sulfite in order to counteract the activity of sulfanilamide and azochloramid.

A typical experiment on the combined bacteriostatic activity of sulfanilamide and azochloramid upon Group A hemolytic streptococcus ensues. Maltose phenol red broths containing sulfanilamide or azochloramid or both (volume 4.8 cc) were inoculated with 0.2 cc of 1 to 10 diluted 24-hour infusion broth culture of hemolytic streptococcus. The number of organisms present were approximately 50,000 per cubic centimeter. The tubes were shaken well and incubated in the dark at 37°C. The resulting growth was noted at various intervals. Contaminations were excluded by preparations of films and subcultures. The results of this experiment are presented in Table I.

TABLE I.  
Combined Bacteriostatic Activity of Sulfanilamide and Azochloramid upon Group A Hemolytic Streptococcus.

Mediums	Hr of incubation				
	8	18	24	48	72
1. Control broth	++	++++	++++	++++	++++
2. Sulfan. (25 mg%) broth	++	+++	++++	++++	++++
3. " (100 " ) "	++	+++	++++	++++	++++
4. Azochl. (1/800,000) broth	—	++	+++	++++	++++
5. " (1/400,000) "	—	++	+++	++++	++++
6. " (1/200,000) "	—	—	—	+++	++++
7. Azochl. (1/800,000)					
8. Sulfan. (25 mg%) broth	—	++	+++	++++	++++
9. Azochl. (1/800,000)					
10. Sulfan. (100 mg%) broth	—	—	—	++	+++
11. Azochl. (1/400,000)					
12. Sulfan. (25 mg%) broth	—	—	—	—	—
13. Azochl. (1/400,000)					
14. Sulfan. (100 mg%) broth	—	—	—	—	—
15. Azochl. (1/200,000)					
16. Sulfan. (25 mg%) broth	—	—	—	—	++
17. Azochl. (1/200,000)					
18. Sulfan. (100 mg%) broth	—	—	—	—	—

— = No visible growth.

+ to ++++ = Various degrees of visible growth.

Table I reveals that with a large inoculum of hemolytic streptococcus the bacteriostatic action of sulfanilamide (25 and 100 mg %) was very slight. Azochloramid in concentrations of 1/200,000 to 1/800,000 exerted some bacteriostatic activity although it failed to prevent entirely the growth of the organisms. The combination of sulfanilamide and azochloramid exerted greater bacteriostatic activity than either compound alone. Used together these two agents in suitable dilutions are more effective than four times the concentration of sulfanilamide alone and twice the concentration of azochloramid alone (Table I). Essentially the same results were obtained in several experiments.

A quantitative study confirmed these results. In one representative experiment the number of viable streptococci per 0.2 cc after 24 hours' incubation at 37° C were as follows: (1) Control broth: 4,000,000; (2) 100 mg % sulfanilamide broth: 2,000,000; (3) 1/800,000 azochloramid broth: 3,000,000; (4) 1/200,000 azochloramid broth: 600; (5) broth containing 25 mg % of sulfanilamide and azochloramid in a dilution of 1/800,000: 2,100. In the same experiment, after 48 hours of incubation, no viable streptococci were demonstrable in broth containing 25 mg % of sulfanilamide and azochloramid in a dilution of 1/400,000, whereas broth containing azochloramid in a dilution of 1/200,000 had 8,000,000 viable streptococci.

Experiments with hemolytic enterococcus revealed that sulfanilamide (1,000 mg %) used in combination with azochloramid (1/800,000) may completely prevent the growth of enterococcus and exert greater bacteriostatic activity than azochloramid alone even in a concentration twice as great.

It remains to be seen whether synergistic effects also may be obtained by the combined use of azochloramid and various sulfamido compounds upon pneumococci and other organisms; furthermore, whether these *in vitro* results may be duplicated *in vivo*.\*

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\* The author wishes to express his appreciation for the supply of azochloramid to Dr. F. C. Schmelkes, Assistant Director, Research Department, Wallace & Tiernan Products.

## Vitamin Deficiency as One Explanation for Inhibition of Protozoan Growth by Conditioned Medium.

R. P. HALL. (Introduced by H. W. Stunkard.)

*From the Biological Laboratory, University College, New York University.*

Accelerative effects of biologically conditioned casein-peptone medium, when added to fresh medium in proportions of 1-5 parts in 10, have been reported previously for *Colpidium campylum*.<sup>1</sup> Subsequently, it has been found that an increase in the proportion of conditioned medium induces inhibitory effects on the growth of *Colpidium campylum* and *Glaucoma piriformis*.<sup>\*</sup> Observations on the latter are presented below.

Series I and II. A culture filtrate was prepared from 96-day flask-cultures of *G. piriformis* in casein-peptone (Difco "tryptone") medium containing  $\text{KH}_2\text{PO}_4$  (1.0 g per l). The cultures were filtered through paper (Whatman No. 12), as previously described by Hall and Loefer,<sup>1</sup> and the filtrate was divided into two portions. One portion (Series I) served as the control medium. To the other (Series II), the following growth-factors were added: Merck's thiamine hydrochloride ( $1 \times 10^{-6}$  g per cc), Merck's riboflavin ( $1 \times 10^{-7}$  g per cc), and S. M. A. Corporation's nicotinic acid amide ( $1 \times 10^{-7}$  g per cc). Each medium was tubed in 10 cc amounts and sterilized in the autoclave at  $122^\circ \text{C}$  for 20 minutes. All tubes received 0.5 cc inocula from a 3-day culture of *G. piriformis* in the basic medium. In both series, the initial pH was 6.9; initial count, 4,000 ciliates per cc. All cultures were incubated in darkness at  $24^\circ \text{C}$ , and counts were made at the indicated intervals (Fig. 1, A).

Series III and IV. The procedure was the same as above, except that 109-day flask-cultures were used as the source of the filtrate, and a 21-day stock culture was used for inoculation. Series III served as the control; in series IV, the medium received the 3 growth-factors. In both series, the initial count was 6,400 ciliates per cc; initial pH, 6.9. The results are described in Fig. 1, B.

In both Fig. 1, A and 1, B the dotted lines represent growth-curves for *G. piriformis* in fresh medium prepared from the same sample of casein-peptone. These series were not run in parallel with Series

<sup>1</sup> Hall, R. P., and Loefer, J. B., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 128.

<sup>\*</sup> In view of the present confusion in nomenclature of these ciliates, it may be pointed out that these 2 strains are known to be antigenically distinct. Consequently, the writer is retaining the older designations for the present.

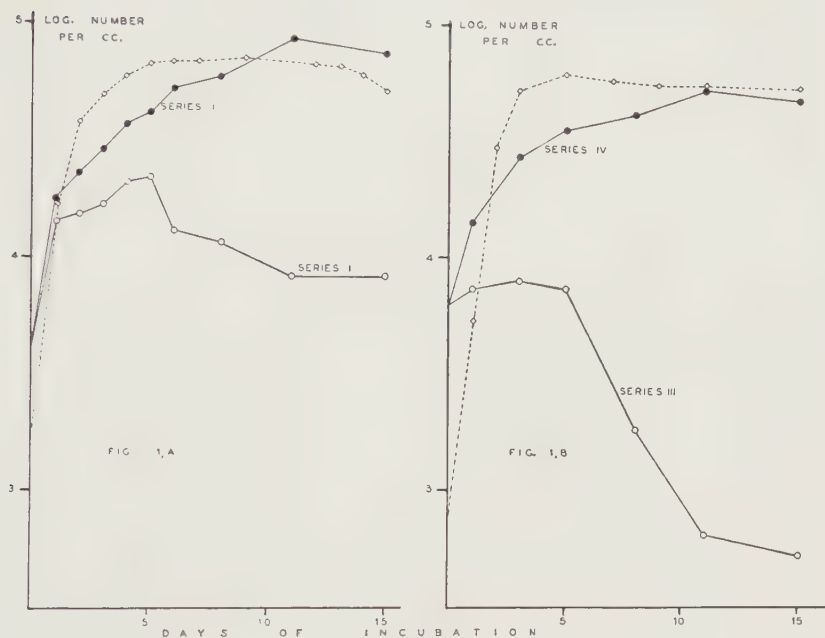


FIG. 1.

I and II, or with III and IV; furthermore, the initial pH was 6.3 instead of 6.9, and the initial counts were lower. However, these series do afford valid comparisons with respect to population yield.

In interpreting the results, it may be pointed out that in all 4 series the medium contained old culture filtrate ("biologically conditioned" medium) in the proportion of approximately 19 parts in 20. In Series I and II, inoculated from a 3-day stock culture, the concentration of any essential materials introduced with the inocula might approach that of fresh peptone medium. For the inocula in Series III and IV, this probably would not be true, since the supply should be depleted to some extent in a 21-day culture. Hence, it might be expected that the differential effect of added vitamins would be greater in Series IV than in Series II. This was actually the case. In both series, however, it is obvious that the addition of 3 growth-factors restored an old culture filtrate to approximately the condition of fresh peptone medium, so far as population yield was concerned.

Comparison of Series I and III suggests that exhaustion of the vitamin supply in cultures of *G. piriformis* is well under way after 21 days, and that inocula from such old cultures carry over no significant amounts of these essential substances. Furthermore, the



results obtained in Series II and IV demonstrate that the concentration of certain vitamins is reduced below the minimum long before the available food supply is exhausted.

These results supplement the earlier report of an accelerating effect produced by old culture filtrates in lower concentrations (Hall and Loefer, *loc. cit.*) The present findings demonstrate that old culture filtrate ("biologically conditioned" medium), in excessive concentration, tends to inhibit growth of *G. piriformis*, with a consequent reduction in population yield. Since this inhibitory effect disappears upon the addition of three growth-factors to the conditioned medium, it may be concluded that the effect actually represents a vitamin deficiency.

### 13121 P

#### Use of Sulfaguanidine in Nutrition Experiments.\*

S. BLACK,<sup>†</sup> J. M. MCKIBBIN AND C. A. ELVEHJEM.

*From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.*

The variable results obtained in studies on the newer members of the vitamin B complex have led us to suspect that intestinal bacteria may synthesize certain unidentified factors essential in the nutrition of the rat. Sulfaguanidine (sulfanilylguanidine), an antibacterial agent which is poorly absorbed from the intestine,<sup>1</sup> appeared to be a useful tool in attacking this problem. The following basal ration was used in our experiments: sucrose 76, purified casein 18, salts 4, corn oil 2, choline hydrochloride 200 mg, nicotinic acid 2.5 mg, calcium pantothenate 2 mg, and .3 mg each of thiamin, pyridoxine and riboflavin. Two drops of haliver oil containing 1 mg of *dl*

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We are indebted to Merck and Company, Rahway, New Jersey, for supplies of thiamin, nicotinic acid, vitamin B<sub>6</sub>, calcium pantothenate; to Abbott Laboratories, North Chicago, Illinois, for haliver oil; to Hoffmann-LaRoche, Inc., Nutley, New Jersey, for dl- $\alpha$ -tocopherol acetate; and to Calco Chemical Division, American Cyanamid Company, Bound Brook, New Jersey, for sulfaguanidine.

<sup>†</sup> Eli Lilly and Company Fellow.

<sup>1</sup> Marshall, E. K., Jr., Bratton, A. Calvin, White, H. J., and Litchfield, J. T., Jr., *Bull. Johns Hopkins Hosp.*, 1940, **67**, 163.

$\alpha$ -tocopherol acetate were given weekly to each rat. Weanling rats were used in all cases.

Sulfaguanidine was fed at levels from 0.5% to 2.0%. At the higher levels the drug is apparently toxic, because a large proportion of the animals died during the first 2 weeks and none grew optimally when given liver extract in addition to sulfaguanidine. Growth results obtained over a 5-week period when the 0.5% level was used are shown in Table I. Sulfaguanidine at a level of 0.5% in the basal ration greatly reduces the growth rate, but when liver extract is included in the diet in addition to 0.5% sulfaguanidine, optimum growth results. Four rats have been maintained for 13 weeks on the basal ration plus 0.5% sulfaguanidine and liver extract, and appear to be normal in every respect; their average weight is 330 g. The addition of 3 mg p-amino benzoic acid per rat per day increased the rate of growth but the rate did not approach that of normal animals.

TABLE I.

Ration	Daily supplement	No. of rats	Weekly growth, g
Basal		5	28.9
Basal + 0.5% sulfaguanidine		10	9.3
" " " "	0.3 g liver extract	8	32.1
" " " "	3.0 mg p-amino benzoic acid	5	18.2

The reduced growth on the basal diet plus 0.5% sulfaguanidine may be due to inhibition of intestinal synthesis of essential growth factors which liver extract supplies, or to a toxicity which is counteracted by a factor in liver extract. Results which indicate that the effect is due to inhibition of intestinal synthesis are shown in Table II. After 3 weeks on the basal ration plus 0.5% sulfaguanidine the animals do not give a growth response when shifted to the basal ration alone, but do respond when supplemented with liver extract. This may be interpreted to mean that the organisms responsible for intestinal synthesis have been eliminated from the intestine and are not reestablished during the first 2 weeks following the removal of sulfaguanidine from the ration. In this connection the results with p-amino benzoic acid are interesting. It has been shown that p-amino benzoic acid reduces the toxic effect of sulfanilamide upon *hemolytic streptococci*.<sup>2</sup> The fact that it produces a growth response when fed with sulfaguanidine from the beginning of the experiment (Table I), and no response during the first week when fed

<sup>2</sup> Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

TABLE II.  
Average Weekly Growth of Rats Receiving Different Supplements Following  
Sulfaguanidine Treatment.  
All rats had received 0.5% sulfaguanidine before being changed.

Ration	No. of rats	Growth	
		1st week g	2nd week g
Continued on basal + sulfaguanidine	11	4.0	0
Changed to basal minus sulfaguanidine	6	-2.0	-0.5
Changed to basal + sulfaguanidine + 0.3 g liver extract daily	4	25.5	25.0
Changed to basal + sulfaguanidine + 3 mg p-amino benzoic acid	4	2.0	12.9

after the animals have received sulfaguanidine for 3 weeks (Table II) may mean that p-amino benzoic acid reduces the toxic effect of sulfaguanidine on certain bacteria in the intestine. When these bacteria are eliminated from the tract before the p-amino benzoic acid is administered, it takes at least a week for the bacteria to become reestablished.

Vitamin K has been shown to be synthesized in the rat's intestine.<sup>3</sup> None of our rats, however, has had a blood clotting time longer than 2 minutes, even after 7 weeks on the sulfaguanidine ration.

*Summary.* It has been shown that on a purified basal ration 0.5% sulfaguanidine greatly reduces the growth rate of young rats. When liver extract is fed with 0.5% sulfaguanidine, optimum growth is obtained. p-amino benzoic acid fed with 0.5% sulfaguanidine from the beginning of an experiment gives a definite growth response, but gives no response during the first week when fed to rats which have received sulfaguanidine alone for several weeks. The bearing of these results upon the possible synthesis of unidentified rat growth factors by intestinal bacteria is discussed.

<sup>3</sup> Brinkhous, K. M., *Medicine*, 1940, **19**, 329.

## 13122

**Delayed Blood Coagulation in Methyl Methacrylate (Boilable "Lucite")\* Vessels.**

JOHN S. HIRSCHBOECK. (Introduced by P. F. Swindle.)

*From the Department of Physiology, Marquette University School of Medicine, Milwaukee, Wis.*

The inhibition of blood coagulation by a plastic resin surface was demonstrated by Neubauer and Lampert.<sup>1</sup> They used a product chemically similar to bakelite which they called "athrombit" and showed that it was superior to paraffin in its ability to delay coagulation. Methyl methacrylate, one of the newer plastic resins, in addition to the properties which make it suitable as a substitute for glass, also possesses an anticoagulant surface.

The anticoagulant power of a surface is usually inversely proportional to the force of adhesion exerted between the surface and water.<sup>2</sup> Methyl methacrylate resin possesses a very pronounced water-repelling surface. The force of adhesion between it and water, when measured by the capillary tube method,<sup>3</sup> is 0.038 g per cm. This is not as low as the paraffin-water nor as high as the glass-water adhesive forces. These are -0.037 g per cm and 0.053 g per cm respectively. Table I presents a comparison of the anticoagulation property of glass, methyl methacrylate and paraffin. The average coagulation time for the series of glass tubes was 6.2 min, the methyl methacrylate tubes 13.9 min and the paraffin tubes 18.3 min. It is evident that the anticoagulant behavior of the methyl methacrylate surface follows Lampert's rule of surface adhesion. In this respect it differs from "athrombit"<sup>2</sup> and collodion.<sup>3</sup>

Clot retraction was delayed in the methyl methacrylate tubes and in some instances did not take place even after standing 24 hours at room temperature.

The tubes used in this experiment had an internal diameter of 1 cm. Two cc of human blood, removed with a dry syringe from the veins of healthy adults, were placed in each tube and the coagulation time was taken when the blood failed to flow upon tilting the tube to the horizontal position.

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\* Supplied for this experiment by E. I. du Pont de Nemours and Company.

<sup>1</sup> Neubauer, O., and Lampert, H., *Muench. Med. Wchnschr.*, 1930, **77**, 582.

<sup>2</sup> Lampert, H., *Die physikalische Seite des Blutgerinnungs-problems*, 1931, Leipzig, Georg Thieme.

<sup>3</sup> Hirschboeck, J. S., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **45**, 122.

TABLE I.  
Coagulation Time of Blood.

Case	Type of surface		
	Glass min	Methyl methacrylate min	Paraffin min
1	7	13	10
2	9	20	27
3	5	9	11
4	5	12	18
5	5	11	19
6	5	14	16
7	7	19	16
8	6	12	18
9	6	11	22
10	7	18	26

*Summary.* The blood coagulation time in methyl methacrylate (boilable "lucite") tubes was found to be twice as long as the coagulation time in glass tubes. The blood coagulation inhibiting effect of this material follows Lampert's rule of surface adhesion.

## 13123

Cleavage of the Imidazole Ring of Histidine and Carnosine  
by Bromine.\*

EDWIN T. MERTZ. (Introduced by A. G. Hogan.)

*From the Department of Agricultural Chemistry, University of Missouri, Columbia.*

The literature records no chemical reagent capable of rapidly splitting the imidazole ring of histidine with the liberation of ammonia. This cleavage is readily accomplished by an enzyme, histidase, found in liver.<sup>1, 2</sup> The following data show that a buffered solution of bromine rapidly splits the imidazole ring of histidine, carnosine and other imidazole compounds.

*Experimental Procedure.* A citrate buffer solution is prepared by dissolving 352.8 g of  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$  in ammonia-free water to a final volume of 1200 cc. The pH is adjusted to 1.0 (glass electrode) by adding approximately 315 cc of C. P. HCl, sp gr 1.18.

\* Contribution from the Department of Agricultural Chemistry, Missouri Agricultural Experiment Station, Journal Series No. 732.

<sup>1</sup> Edlbacher, S., and Kraus, J., *Z. Physiol. Chem.*, 1930, **191**, 225; 1931, **195**, 267.

<sup>2</sup> Edlbacher, S., and Neber, M., *Z. Physiol. Chem.*, 1934, **224**, 261.



Liquid C. P. bromine is then added in excess to 100 cc of the buffer solution, the mixture is shaken, and allowed to stand for 12 hours. This saturated bromine solution (approximately 1.0 N bromine) is kept in the dark, and is prepared fresh every fourth day.

To determine the ammonia liberated by bromination, 1 cc of a solution containing 0.5-2.0 mg of imidazole nitrogen is placed in a 25 x 200 mm test tube with 9 cc of the bromine buffer solution. The tube is covered with a 40 x 60 mm test tube and protected from light for a period of 2 hours.<sup>†</sup> An excess of 20%  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  solution<sup>‡</sup> is then added. At the end of 10 minutes a few drops of motor oil are added, the tube is connected with an aeration apparatus, an equal volume of approximately 15 N NaOH solution is introduced, and the alkaline imidazole solution is aerated until it is free of ammonia (30-60 minutes). The ammonia is trapped in 2%  $\text{H}_3\text{BO}_3$  solution containing methyl red-methylene blue indicator, and is titrated with 0.01 N HCl.<sup>§</sup>

*Results.* The volatile amine obtained by the above procedure was identified as ammonia by converting it to benzamide; 0.24 g of pure benzamide was recovered from 0.5 g of histidine monohydrochloride. The ammonia was also identified by quantitative colorimetric comparisons with nesslerized ammonium sulfate.

The yield of ammonia from brominated histidine is dependent upon 4 factors: pH of the solution at the time of bromination, concentration of bromine, the period of bromination, and the period of reduction. The influence of each factor was determined separately and the optimum conditions were found to be as follows: pH: 1-1.5, bromine concentration: 0.5-1.0 N, period of bromination: 2 hours or more, and period of reduction: 3 minutes or more. Under these *optimum* conditions, 80( $\pm 2$ )% of the total nitrogen of histidine was recovered as ammonia at every level of histidine monohydrochloride tested (3, 6, 9, and 12 mg).

The ammonia recoveries obtained under these optimum conditions from related imidazole compounds are listed in Table I.

The extent of liberation of ammonia from the imidazole ring is affected by the nature of the side chains attached to the ring. Imidazole itself does not yield all of its nitrogen as ammonia. Methylation

<sup>†</sup> Tests on histidine show that under these conditions the bromination reaction is 90% complete in 5 minutes, 95% complete in 15 minutes, and 100% complete in 1 hour. At a concentration of 0.5 N bromine, the reaction is 100% complete in 2 hours.

<sup>‡</sup> Excess bromine can be removed equally effectively with  $\text{Na}_2\text{S}$ , or by aeration. The use of  $\text{Na}_2\text{S}$  is more convenient.

<sup>§</sup> Sobel, A. E., Yuska, H., and Cohen, J., *J. Biol. Chem.*, 1937, **118**, 443.

TABLE I.  
 The Ammonia Recovery from Various Imidazole Compounds.

Compound	Quantity used mg	Total nitrogen (theory) mg	Ammonia nitrogen found mg	Recovery of imidazole nitrogen %
Imidazole	4.84	2.00	1.096 1.094	54.7
2-methyl imidazole	3.41	1.16	0.632 0.640	54.8
4 (or 5)-methyl imidazole oxalate	6.65	1.12*	0.348 0.338	30.6
Imidazole lactic acid monohydrate	6.00	0.96	0.962 0.973 0.937	99.7
Histamine dihydrochloride	4.38	1.00	0.083 0.091	13.0
1-carnosine	8.07	2.00	0.897 0.899	89.8
1-carnosine + 4.2 mg CuCO <sub>3</sub> · Cu(OH) <sub>2</sub>	8.60	2.13	1.062	100.6
1-carnosine + 50 mg CuCO <sub>3</sub> · Cu(OH) <sub>2</sub>	8.70	2.16	1.097	
Cu-carnosine	10.91	1.87†	0.955 0.926 0.931	100.2
Cu-anserine	11.41	1.88‡	0.273 0.261	28.4
Acetyl l-histidine	10.70 10.90 9.60	2.09 2.13 1.87	1.286 1.325 1.178	93.4
Acetyl l-histidine + 50 mg CuCO <sub>3</sub> · Cu(OH) <sub>2</sub>	10.10 10.55	1.97 2.06	1.298 1.355	98.8
l-histidine monohydrochloride monohydrate	10.10	2.02	1.600	118.9
l-histidine monohydrochloride monohydrate + 50 mg CuCO <sub>3</sub> · Cu(OH) <sub>2</sub>	10.30 10.00	2.06 2.00	1.640 1.580 1.570	118.6
Ergothionine	5.45	1.00	0.198 0.219	31.2

\*Total nitrogen found (micro-Kjeldahl). Theory for C<sub>4</sub>H<sub>6</sub>N<sub>2</sub> · H<sub>2</sub>C<sub>2</sub>O<sub>4</sub> = 1.08 mg.

†Total nitrogen found (micro-Kjeldahl). Theory for C<sub>9</sub>H<sub>14</sub>O<sub>3</sub>N<sub>4</sub> · CuO = 2.00 mg.

‡Total nitrogen found (micro-Kjeldahl). Theory for C<sub>10</sub>H<sub>16</sub>O<sub>3</sub>N<sub>4</sub> · CuO = 2.00 mg.

of the 2-position has no apparent effect on ammonia recovery, whereas methylation of the 4 (or 5) position decreases it. A marked decrease is also observed when the β-ethyl amine group is in the 4 (or 5) position of the ring (histamine). On the other hand, all of the ring nitrogen is recovered as ammonia when a lactic acid group is in the 4 (or 5) position of imidazole (imidazole lactic acid).

l-Carnosine and acetyl l-histidine yield 7-10% less ammonia than the values calculated for complete cleavage of the imidazole ring.

However, if cupric ion is added at the time of bromination, the ammonia recovery is 100%. The action of cupric ion may be similar to its well-known catalytic effect on Kjeldahl digestions.

Under optimum conditions of bromination, histidine yields 2.4 moles of ammonia per mole of compound. Attempts to modify the experimental conditions so as to recover either exactly 2 or 3 moles of ammonia have never been successful, and cupric ion has no effect upon ammonia recovery (Table I). The additional 0.4 mole of ammonia obtained from one mole of histidine may be derived from a partial cleavage of the alpha-amino group.

*Summary.* When histidine and related compounds are treated with bromine at pH 1 to 1.5, the excess bromine removed, and the solution alkalinized and aerated, significant amounts of ammonia are recovered from the imidazole ring. Copper carnosine, copper acetyl histidine, and imidazole lactic acid yield amounts of ammonia which correspond to a complete cleavage of the ring nitrogen. Histidine yields sufficient ammonia to account for all of the ring nitrogen plus almost one-half of the alpha-amino nitrogen. Other compounds tested gave 13-55% of their imidazole nitrogen as ammonia.

### 13124

#### A Simple Method for the Bioassay of Renin.

OTTO SCHALES AND FLORENCE W. HAYNES. (Introduced by Soma Weiss.)

*From the Medical Clinic, Peter Bent Brigham Hospital, and the Department of Medicine, Harvard Medical School, Boston.*

In connection with work on hypertension it was of importance to have a simple method for testing a large number of extracts for their renin content. Rabbits were used for the test as their blood pressure is conveniently measured in the artery of the ear by a membrane manometer.<sup>1</sup>

After several preliminary trials a procedure was standardized, which, despite its simplicity gives reliable and reproducible results, as accurate as one would expect from a bioassay.

The test was carried out in a warm room. The unanesthetized animals were placed in a box with open front and top; after some

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<sup>1</sup> Grant, R. I., and Rothschild, P., *J. Physiol.*, 1934, **81**, 265.

training they would sit quietly. The left ear was placed between the capsule and light of the manometer without forcing it away from its natural position. Measurements of the blood pressure were taken at about  $\frac{1}{2}$ -minute intervals; after a few minutes the readings were constant, fluctuating not more than one millimeter from the average level. After a constant level had been maintained for at least 3 minutes, the extract was injected intravenously in the right ear. The introduction of the needle had no effect on the blood pressure, as the right ear had previously been denervated. Injection of 5 cc of physiological saline solution also had no effect on the blood pressure.

The injection time chosen was 60 seconds, but practically the same results were obtained if the injection was made in 30 seconds. When the injection was started, measurements of the blood pressure were done in rapid succession, about 3 per minute. The maximum response to renin was usually obtained about 3 minutes after the start of the injection. For each extract 4 animals were used and amounts of renin were injected which would cause a rise in blood pressure of 20 to 40 mm mercury. If the potency of an extract was unknown, this amount was first determined approximately by an experiment with one animal. The volume of isotonic solution injected (0.9% NaCl) was kept between 1 and 2 cc per rabbit.

From the data on 4 animals the amount of renin was calculated (as micrograms nitrogen) which had to be given per kilogram of rabbit to cause an average rise in blood pressure of 30 mm. We call this amount one rabbit unit (R.U.). It is the number derived from the average response of 4 animals and its calculation is justified by our finding that the response in the range of 20 to 40 mm pressure rise is approximately proportional to the amount of renin given. This straight linearity is not present in the range above 40 mm as one can see in the dose-response curve by Pickering and Prinzmetal.<sup>2</sup> With increasing purity of an extract the amount of nitrogen equivalent to one rabbit unit will decrease as less inert proteins are present and the method offers therefore a simple way to judge the success of any attempt to purify renin. The degree of purity can be expressed as the number of rabbit units per milligram of nitrogen. Independent of its purity the concentration of renin in an extract may be expressed as the number of units per cc.

The following data illustrate the assay procedure. Table I shows the bioassay of an extract, repeated after an interval of 2 days. To calculate one rabbit unit, the sum of micrograms of nitrogen given

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<sup>2</sup> Pickering, G. W., and Prinzmetal, M., *Clin. Sc.*, 1938, **3**, 211.

TABLE I.  
Extract N55 (diluted 1:25, 1 cc equals 41.6  $\mu\text{g}$  nitrogen).

Rabbit No.	$\mu\text{g}$ N <sub>2</sub> given per kg 2/18/41	Rise in B.P. mm Hg	$\mu\text{g}$ N <sub>2</sub> given per kg 2/20/41	Rise in B.P. mm Hg
42	50.8	27		
59			46.0	18
64			50.3	37
65	30.0	18	48.5	33
66	45.5	28		
68	39.6	27	44.4	30
	165.9	100	189.2	118
$1 \text{ R.U.} = \frac{165.9 \times 30}{100} = 49.8 \text{ } \mu\text{g N}_2/\text{kg.} \quad \frac{189.2 \times 30}{118} = 48.1 \text{ } \mu\text{g N}_2/\text{kg.}$				
$1 \text{ cc (undiluted)} = \frac{1040}{49.8} = 20.9 \text{ R.U.} \quad \frac{1040}{48.1} = 21.6 \text{ R.U.}$				

per kilogram is divided by the sum of the rises in blood pressure and multiplied by 30.

Table II shows the result of the bioassay of 2 preparations of renin, C<sub>1</sub> and C<sub>2</sub>, made according to the method of Helmer and Page<sup>3</sup> for their fraction C. From the statement of the authors one can conclude that an amount of this fraction containing 93  $\mu\text{g}$  of nitrogen caused a rise in blood pressure of 30 mm or more per kilogram of dog. The findings in Table II confirm the statement of Hessel<sup>4</sup> that about the same amount of renin was necessary per kilogram to produce the same rise in blood pressure in rabbits as in dogs.

The accuracy with which this method can be used to determine yields can be seen from the following example:

TABLE II.  
Extract C<sub>1</sub> (diluted 1:50, 1 cc equals 78.4  $\mu\text{g}$  nitrogen).  
" C<sub>2</sub> ( " " 1:50, 1 " " " 93.6 " " " ).

Rabbit No.	Extract C <sub>1</sub>		Extract C <sub>2</sub>	
	$\mu\text{g}$ N <sub>2</sub> given per kg	Rise in B.P. mm Hg	$\mu\text{g}$ N <sub>2</sub> given per kg	Rise in B.P. mm Hg
52	125	34	127	42
55	127	40	127	36
56	130	21	126	32
57	130	31	130	20
	512	126	510	130
1 R.U.	122 $\mu\text{g}$ N <sub>2</sub>		118 $\mu\text{g}$ N <sub>2</sub>	

<sup>3</sup> Helmer, O. M., and Page, I. H., *J. Biol. Chem.*, 1939, **127**, 757.

<sup>4</sup> Hessel, G., *Klin. Wchnschr.*, 1938, **17**, 843.



A solution containing 12400 rabbit units was fractionated and the resulting precipitate was found to contain 650 R.U. The assay of the filtrate showed it contained 11700 R.U. This is a total recovery of 99.6%. In working with small quantities of concentrated solutions, small unavoidable losses are incurred in the various procedures. For example, a solution containing 3100 R.U. gave 2 fractions with 2740 and 10 R.U. The recovery in this case was 88.7%.

The simple method for the bioassay of renin, suggested in this paper, will make it possible to give data concerning yield and degree of purity for each step of the various chemical procedures involved in the preparation of renin.

### 13125

#### Distribution of Body Weight in the Organs and Tissues of the Rabbit.\*

CHARLES J. LEVINE, WALTER MANN, HAROLD C. HODGE, I. ARIEL  
AND O. DUPONT.

*From the Departments of Radiology and Biochemistry and Pharmacology, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.*

In the study of the distribution of radioactive isotopes in rabbits, it is usually convenient to prepare for the counting procedures, 1 or 2 g or less of the various tissues. From this sample, the percentage of the isotope dose per gram of tissue may be calculated. However, to account for all of the isotope dose, it becomes necessary to know the total amount of muscle, brain, bone and other tissues in the experimental animal. Such data are not available in the literature.

*Procedure.* The animals were adult, male, New Zealand albino rabbits in good health which had been for several months on our stock ration of oats, alfalfa and lettuce supplemented by chow.

Immediately upon sacrifice, the skin was removed from the body. Considerable subcutaneous fat adhered to the skin and was not separated from it.

The viscera weight included the usual viscera, the salivary glands, larynx, perirenal fat and also the weight of blood removed in the blood volume determinations.

All the adhering soft tissues were removed from the skeleton. As

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each bone was cleaned, it was placed in tap water. When all the bones were prepared, they were dried with blotting paper and weighed together. The weight of the muscle was obtained by difference.

The fresh bones were dried overnight at 105°C, the fat removed by alcohol extraction, and the extracted bones dried on a steam bath. The remaining organic matter was removed by boiling in 3% KOH solution in ethylene glycol, followed by washing in boiling water. The inorganic residue was dried overnight at 105°C.

*Discussion.* The brain weight expressed as a percentage of the body weight was remarkably constant (about 0.3%) (Table I). The bone percentage was fairly constant. The other tissues (see also Table III) showed some variability but in no case did the average deviation exceed 6%. These data provide the desired basis for the calculation of the distribution of radioactive isotopes in the rabbit.

The distribution of bone constituents for the entire skeleton (Table II) gives a somewhat different picture than is usually obtained from analyses of femur or humerus. The average values expressed on the wet bone weight basis are as follows: water, 38%; fat, 9%; other organic matter, 20%, and ash, 33%. The organic to inorganic ratio is not much different from femur analyses, but the water content is high. This may have been an artifact contributed by the method of storing the bones.

TABLE I.  
Percentages of Blood, Skin and Fur, Viscera, Bone, Brain and Muscle in the Rabbit.  
Data are given as percentage of intact body weight.

Rabbit No.	Rabbit wt, g	Blood Vol.,* %	Skin and Fur, %	Viscera, %	Wet Bone, %	Brain, %	Muscle, %	Recovery, %
1	3420		13.3	22.0	8.2	0.32	56.3	99.8
2	3440	7.4						
3	2935		13.9	27.1	8.4	0.32	50.4	99.8
4	3160		13.3	25.5	7.8	0.32	53.2	99.8
5	3050	6.8	13.2	27.8	8.6	0.33	50.4	100.0
6	3615	6.9	14.9	24.0	7.5	0.30	53.5	99.9
7	2630	8.2						
8	3560	6.1	13.1	26.1	8.5	0.30	52.3	100.0
9	3590	6.3						
10	3045		14.5	26.5	7.7	0.30	50.5	99.2
11	2655		15.0	26.8	7.2	0.34	51.0	100.0
12	2630		11.9	29.8	8.6	0.37	49.4	99.7
13	2445		11.2	26.9	8.1		53.2	99.4
Avg	3090	7.0	13.4	26.2	8.0	0.32	52.0	
Avg deviation		0.6	0.9	1.5	0.4	0.02	1.7	

\*The blood volume figures are not a part of the "Recovery" column.

TABLE II.  
Distribution of Weight Between the Various Components of Rabbit Bone.

Rabbit No.	Wet wt, g	Dry wt, g	Fat, g	Total organic material, g	Ash, g
1	279	182	28.0	86.0	96.0
3	248	148	19.1	65.5	82.8
4	246	156	24.0	73.5	82.5
5	251	156	24.3	77.8	77.9
6	270	172	32.0	79.4	92.8
8	302	176	32.0	91.2	84.6
11	190	119	14.5	56.5	62.5
12	225	138	17.0	61.7	76.3
13	198	120	17.5	56.0	64.0
Avg	245	152	23.2	71.9	79.8
Avg deviation	27	18	5.4	10.7	8.7

TABLE III.  
Weights of Certain Organs in the Rabbit.\*

Rabbit body wt, kg	Kidney, g	Spleen, g	Adrenal, g	Heart, g	Lung, g	Submaxillary salivary gland, g	Liver, g	Thyroid, g
2.90	23	0.6	0.50	11.2	12	1.4	120	
3.12	20	1.5	0.45	10.0	32	1.5		0.35
2.40	20	1.2	0.20	9.0	18	2.4	150	0.20
2.67	20	1.0	0.45	8.8	16	1.6	75	0.30
3.40	24			9.4	25	1.8	112	0.35
3.00	21	2.7	0.50	9.1	13	1.6	106	0.25
3.35	25	2.0	1.15	9.3	15	1.9	84	0.55
2.88	18	2.2	0.75	7.6	12	1.4	84	0.40
3.20	21	1.8	0.60	15.8	27	1.8	133	0.50
3.55	24		0.45	10.8	19	1.4	117	0.35
3.00	16	1.4	0.40	7.5	14	1.3	146	0.20
2.81	22	1.4	0.55	10.6	14	1.3	137	0.71
2.45	18	1.7	0.45	7.1	14	1.0	97	0.60
2.25	13	1.0	0.73	5.8	12	1.0	66	0.55
2.64	20	1.6	0.35	8.3	11	1.2	116	0.24
2.72	22	1.2	0.35	7.2	24	1.1	107	0.42
2.75	17	2.0		8.8	22	1.9	108	0.50
2.03	24	1.2	0.25	8.6	15	1.5	98	0.55
2.82	11	2.5	0.50	10.5	12	1.2	88	0.30
2.71	25	0.7	0.50	8.3	20	1.5	89	0.30
3.35	24	1.5	0.30	7.6	16	1.6	98	0.22
3.27	24	3.5	0.47	10.6	21	1.3	97	0.50
3.51	26	0.9	0.40	13.1	37	1.7	120	0.55
2.94	17	1.5	0.35	8.2	18	1.8	102	0.35
3.50	20	2.4	0.45	9.2	22	1.8	114	0.45
2.66	24	2.2	0.40	6.0	12	1.3	83	0.50
2.84	18	2.5	0.85	8.2	13	0.8	108	0.10
2.99	23	2.4	0.25	7.6	18	1.4	91	0.25
3.02	20	1.5	0.20	8.4	17	1.4	122	0.20
2.90	21	2.4	0.35	8.3	15	1.2	103	0.35
2.98	19	1.7	0.26	7.2	11	1.0	91	0.50
Avg 2.92	21	1.7	0.46	9.0	18	1.5	105	0.39
Avg deviation 0.28	3	0.5	0.14	1.4	5	0.3	16	0.12

\*The distribution of radioactive arsenic in these tissues is given in a report by Ariel, I., DuPont, O., and Warren, S. L., *J. Syph. Gonn.*, in press.

*Summary.* Ten adult, male, New Zealand albino rabbits were used to determine the percentages of body weight of blood, skin and fur, viscera, bone, brain and muscle. The brain weight is a constant percentage of body weight, the bone weight fairly constant and the other tissues vary within 6%.

## 13126

Transitory Diminution of Blood Pyruvate *in vitro*.

JEROME S. HARRIS AND SAMUEL E. ELGART. (Introduced by W. A. Perlzweig.)

*From the Department of Biochemistry, Duke University School of Medicine, Durham, N.C.*

In freshly drawn blood with or without added pyruvate, there occurs first a rapid fall in pyruvate content followed by a later rise.<sup>1, 2</sup> These changes are abolished by the addition of iodoacetate.<sup>2</sup>

In order to determine whether serum or blood cells are responsible for the fall in pyruvate content, freshly drawn human blood was defibrinated with glass beads and the serum was separated by centrifugation. The pyruvate content was determined by Lu's method<sup>3</sup> modified by the use of tungstic acid protein precipitation. Blood cells suspended in saline caused a rapid change in added pyruvate while the serum had no effect (Table I).

The rate and extent of this reaction varied with the blood cell suspensions of different subjects. It was not affected by anaerobiosis with carbon monoxide or nitrogen.

TABLE I.

Protocol: Blood was defibrinated and the serum separated. Red cells were washed once in 0.85% NaCl and resuspended in Ringer-PO<sub>4</sub> Buffer (0.02 M, pH 7.3) to 50% hematocrit. Both serum and cells after pyruvate addition kept at 37.5° in water bath with constant slow shaking.

	Pyruvate added, μg/ml	Theoretical content, μg/ml	Pyruvate found, μg/ml (Time after addition of pyruvate)				
			1 min.	10 min.	30 min.	120 min.	300 min.
Serum	83.4	84.1	84.2	83.9	84.0		84.0
Cells	21.6	22.3	12.7	8.2	7.8	23.3	

<sup>1</sup> Wilkins, R. W., Weiss, S., and Taylor, F. H. L., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **38**, 296.

<sup>2</sup> Bueding, E., and Wortis, H., *J. Biol. Chem.*, 1940, **133**, 585.

<sup>3</sup> Lu, G. D., *Biochem. J.*, 1939, **33**, 249.

TABLE II.  
Comparison of the Recovery of Pyruvate in Hemolyzed and Unhemolyzed Blood Cell Suspensions.

	% recovery (Time after addition of pyruvate)	
	1 min.	10 min.
Blood No. 1—Cell suspension	37.8	21.0
Cell suspension hemolyzed with saponin	5.3	67.5
Blood No. 2—Cell suspension	62.4	
Cell suspension hemolyzed by freezing and thawing	40.0	

When the blood cell suspensions were hemolyzed, either by the addition of saponin or by alternate freezing and thawing, the fall in pyruvate content after the addition of pyruvate occurred more rapidly and to a greater extent than in the case of the unhemolyzed cell suspensions (Table II).

These differences are probably due to the necessity for penetration of the pyruvate into the cells. This can be demonstrated by analysis of the fluid and cell portions of a suspension of blood cells after the addition of pyruvate. There was an immediate rise in pyruvate content of the fluid portion (obtained by centrifugation) followed by a slow fall. The cells showed very little rise during this time. Apparently, as the pyruvate entered the cells, it reacted and could not be recovered. Although the system or substance responsible for this change is stable in the intact cell at room temperature, hemolyzed cells which had remained at room temperature for one hour failed to show the fall in pyruvate content after the addition of pyruvate. Cells hemolyzed by the addition of dilute sulfuric acid (0.074 N) were also ineffective.

Bueding and Wortis<sup>2</sup> have attributed the rapid fall in pyruvate content of freshly drawn blood to the cocarboxylase content of that blood. In order to test this hypothesis, the (pooled) blood cell suspension of rats (maintained on a vitamin B<sub>1</sub> deficient diet until marked weight loss and polyneuritis occurred) was divided into two portions. To one was added cocarboxylase\* (final concentration 3.3 mg %). After 10 minutes' incubation at 37.5°C, sodium pyruvate (23  $\mu$ g pyruvic acid per ml) was added to both cell suspensions. A drop of 44% occurred in the sample with added cocarboxylase as against 39% for the control. In order to eliminate the influence of cocarboxylase penetration of the cells, the same experiment was repeated using hemolyzed cell suspensions without any

\* Grateful acknowledgment is made to Merck and Company, Rahway, New Jersey, for the cocarboxylase used in these experiments.



significant differences in the result. As a further control, rats were placed on the same deficient diet with the addition of 20 mg thiamin chloride daily for an identical period of time. The pooled blood cell suspension showed a drop of 34% after addition of 27  $\mu$ g pyruvic acid per ml, which is in agreement with the data obtained with the blood of the deficient rats. The hematocrit value of all blood suspensions was 50%. Hence, it is apparent that drop in blood pyruvate is independent of the cocarboxylase content.

In an attempt to determine the fate of the pyruvate, the oxygen consumption and the carbon dioxide production were studied by means of the Warburg technic.<sup>4</sup> In spite of the disappearance of 71 and 65  $\mu$ g pyruvic acid in 2 separate experiments, no significant changes in oxygen or carbon dioxide pressures were observed.

In order to determine whether the pyruvate is converted to some other ketonic acid *in vitro*<sup>1</sup> a number of experiments were performed.

7.7 mg sodium pyruvate (equal to 6.16 mg pyruvic acid) were added to 80 ml of blood cell suspension containing 0.26 mg pyruvic acid. After 10 minutes' incubation at 37.5°C a sample was taken for ordinary analysis (Lu) and the remainder was utilized for the isolation of the hydrazone. The total pyruvic acid present at the start was 6.4 mg, and 1.03 mg or 16% was found by the method of Lu after incubation for 10 minutes. Three mg of the pure 2,4-dinitrophenylhydrazone of pyruvic acid was recovered gravimetrically corresponding to 0.99 mg pyruvic acid. The hydrazone melted at 213-15°C (uncorr.), did not depress the melting point of synthetic 2,4-dinitrophenylhydrazone of pyruvic acid, and gave the theoretical content of pyruvic acid by Lu's method. Accordingly, gravimetric analysis indicates that the loss is due to an actual disappearance of pyruvic acid, and not due to the conversion of some other keto acid which might have a smaller color value in Lu's method.

Total keto acids were assayed by the method of Lu, extracting the hydrazone with 15% sodium carbonate and developing the color with 3 N sodium hydroxide. The total ketonic acids always check with the pyruvate determinations even when 60% of the added pyruvate had disappeared.

Methyl glyoxal, glyoxal, diacetyl (as the 3-nitro-benzohydrazides),  $\beta$ -ketonic acids (method of Edson<sup>5</sup>), and acid and alkali hydrolysable derivatives of pyruvic acid could not be demonstrated.

Determinations of lactic acid<sup>6</sup> revealed no change during an in-

<sup>4</sup> Dixon, M., *Manometric Methods*, Cambridge University Press, 1939.

<sup>5</sup> Edson, N. L., *Biochem. J.*, 1935, **29**, 2082.

<sup>6</sup> Elgart, S., and Harris, J. S., *Ind. Eng. Chem., Anal. Ed.*, 1940, **12**, 758.

terval when the pyruvic acid content decreased from 5.75 to 3.58 mg %. In another experiment the lactic acid rose 0.4 mg % while the pyruvate concentration fell 2.54 mg %. This is in accord with the findings of Flock, Bollman, and Mann<sup>7</sup> that sodium pyruvate incubated with blood does not cause any change in lactic acid content.

It has been reported<sup>2</sup> that potassium cyanide increases the magnitude of the disappearance of pyruvate due to a potentiation of cocarboxylase action. However, as we have already demonstrated, the disappearance of pyruvate does not depend upon cocarboxylase content. Furthermore, the addition of the same amount of cyanide (final concentration of 0.5% sodium cyanide) to standard solution of pyruvate or to serum as well as to blood containing added pyruvate results in an almost complete disappearance of pyruvate. This may occur either from polymerization of the pyruvate or from cyanohydrin formation.

*Conclusions.* 1. The disappearance of pyruvate added to blood *in vitro* is caused by the blood cells and not the serum. 2. Hemolysis of the cells by saponin or freezing increases the speed and extent of this reaction. 3. The extent and rapidity of the reaction is not altered by Vitamin B<sub>1</sub> deficiency of the cell donor, nor by the addition of cocarboxylase to the cells *in vitro*. 4. Anaerobiosis has no effect on the reaction. 5. The reported effect of cyanide upon the reaction is due to an artefact. 6. The pyruvate which disappears is neither decarboxylated nor changed to other ketonic acids or lactic acid.

## 13127

### Electro-Magnetic Measurement of Blood Flow and Sphygmomanometry in the Intact Animal.

A. KOLIN, J. L. WEISSBERG AND L. GERBER. (Introduced by B. S. Oppenheimer.)

*From the Laboratories, Mount Sinai Hospital, New York City.*

The electro-magnetic method of measuring blood flow<sup>1</sup> has been discussed in detail in previous publications.<sup>2, 3</sup> In this communication we wish to describe a modification which we believe to be

<sup>7</sup> Flock, E., Bollman, J. L., and Mann, F. C., *J. Biol. Chem.*, 1938, **125**, 49.

<sup>1</sup> Kolin, A., *PROC. SOC. EXP. BIOL. AND MED.*, 1936, **35**, 53.

<sup>2</sup> Katz, L. N., and Kolin, A., *Am. J. Physiol.*, 1938, **122**, 788.

<sup>3</sup> Kolin, A., *PROC. SOC. EXP. BIOL. AND MED.*, 1941, **46**, 235.

Additional references may be found in 3.

more widely applicable to studies in vascular physiology since it permits blood flow measurements in the recovered animal over an extended period of time\* and combines with it a method of sphygmomanometry.

The principle of this method is based on the fact that an electromotive force is induced in the stream of blood as it moves across a magnetic field. This e.m.f., which is proportional to the rate of flow, is amplified and photographically recorded. With the exception of the magnet the apparatus utilized in the present modification is the same as described in reference 3.

In the present modification a small snugly fitting rubber sleeve (R:Fig. 1) containing the pick up electrodes is implanted about the given vessel using sterile precautions. The wound is closed and the animal is permitted to recover. Thereafter blood flow measurements may be performed in the intact animal by placing the desired part in the gap of a large a.c. electro-magnet. The size and shape of the magnet which we employed permitted observation in anesthetized animals in somewhat unnatural positions. However, with a suf-

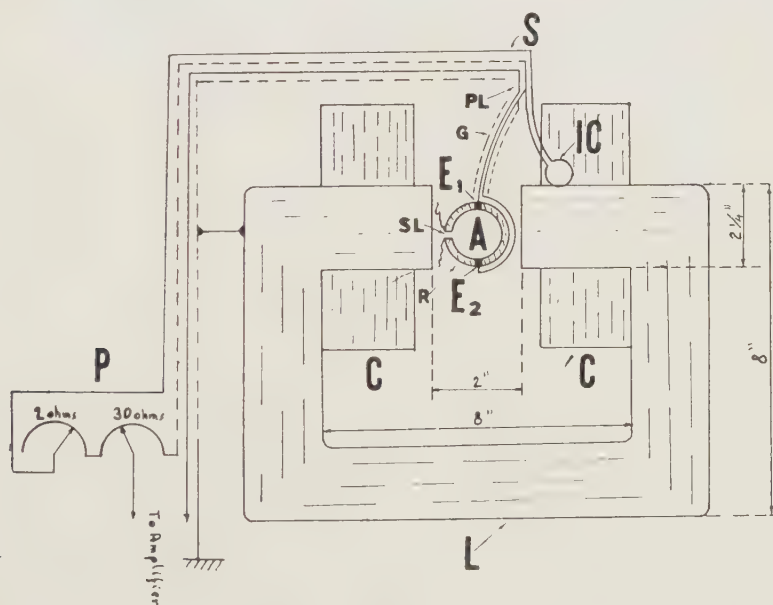


FIG. 1.

Cross-section of electro-magnet and potentiometer circuit. P: compensating potentiometer. CC: coils. L: laminated iron core. IC: induction coil. S: insulated wire. R: pick up sleeve (greatly exaggerated in size). A: artery. E<sub>1</sub> and E<sub>2</sub>: electrodes. SL: slit. G: grounded, flexible shield. PL: location of plug.

\* Thus far our experiments were terminated after a period of 3 days because of thrombosis on subsequent handling of the vessel.

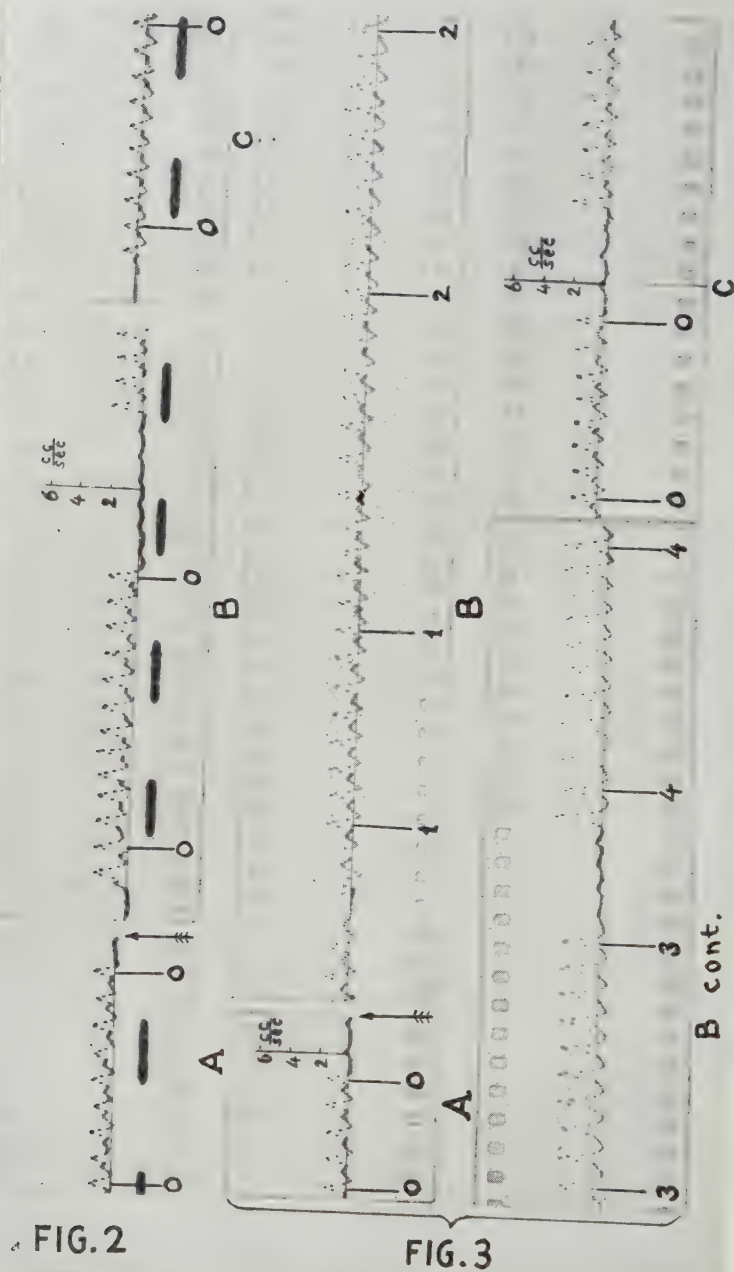


Fig. 2. Effect of 60  $\gamma$  of adrenalin on femoral flow (given intravenously). Time intervals 2.4 sec. (Table I.)  
 Fig. 3. Effect of 25 mg of papaverin on femoral flow (given intravenously). Film speed the same as above. Arrows indicate injection of the drug. (Table I.)

ficiently large magnetic gap properly arranged animals could be placed in comfortable positions and blood flow could be recorded without resorting to anesthesia.

*Method.* The blood velocity is recorded with a cathode ray oscilloscope. Its deflection is linearly proportional to the rate of flow. Since the changes of flow are followed without inertia one can easily distinguish between the systolic and diastolic velocities (Figs. 2 and 3). The recorded curve consists of dots which are spaced at intervals of  $1/60$  of a second when the magnet is fed with a 60 cycles a.c. A greater continuity of the record can be obtained either by slowing down the recording film<sup>3</sup> or by using an alternating magnetic field of a higher frequency.

The calibration of the instrument reading in terms of cc/sec was performed in all cases by perfusing the excised blood vessel with blood and measuring the outflow with a graduate and stop watch. For observation of relative changes of blood flow a calibration is unnecessary. The average flow during a given time interval can be determined by planimetry.<sup>2</sup> The area of the curve below the base line, corresponding to zero flow, is to be considered as negative.

The method was also adapted for simultaneous determination of blood pressure<sup>†</sup> by placing minute rubber cuffs, patterned after the fashion of the sphygmomanometer, on either side of the pick up sleeve. The air pressure within the cuffs is measured and raised until the systolic flow pulsations disappear in the oscilloscope. The corresponding manometer reading gives the systolic blood pressure. We compared pressure readings as obtained by this method in the left femoral artery of a dog with pressures determined simultaneously in the cannulated right femoral artery and found the results to check within 3%.

Fig. 1 shows diagrammatically the a.c. magnet with the rubber sleeve, R, placed around an artery A (greatly exaggerated in size) and the compensating potentiometer, P. The rest of the circuit is exactly as described previously.<sup>3</sup> The peak value of the magnetic field strength was 500 Oersted. The coils, CC, consisted of 300 turns each and passed 9 amperes when connected in parallel to the 50 volt terminals of a step down transformer. IC is a small coil which supplies the compensating voltage across the potentiometer, P. The potentiometer is placed next to the amplifier several yards away from the magnet. The connections to the potentiometer are made through a single conductor, S, and an "air-plane cable" whose grounded outer conductor is indicated by dotted lines. The voltage

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† Technic devised by J. L. Weissberg.



picked up by the electrodes  $E_1$  and  $E_2$  is conducted to the amplifier by 2 insulated flexible wires which terminate in a small plug, PL. We used enameled cotton covered wires which were held together by a coating of "Vultex". A grounded spiral G (1.8 mm external diam) wound of thin copper wire was placed around the lead wires. A coating of "Vultex" was applied to its outside as well as to the rubber sleeve, R, so that all metal parts were thoroughly imbedded in rubber. The sleeve, R, is a section of a catheter about 4 mm long. The contact electrodes,  $E_1$  and  $E_2$ , were made by removing the insulation at the end of the lead wires and threading them through 2 stitches into the wall of the rubber tubing. The slot S L was closed by tying the silk threads attached to its edges. We prepared sleeves of various sizes for different diameters of the artery A which is slightly compressed when the sleeve is tied around it.

*Pharmacological Illustrations of the Method.*<sup>‡</sup> Figs. 2 and 3 represent the velocity curves obtained from the left femoral artery of a 50-lb dog after intravenous nembutal anesthesia 1½ days after the implantation of the pick up and compression units. The base line corresponding to zero flow was checked from time to time by occluding the artery with a compression cuff distal to the pick up sleeve. Owing to incomplete occlusion there were small systolic peaks in the base line. The base line was consequently plotted in coincidence with the diastolic level. Average flows were obtained by planimetry of sections of an enlarged record (Table I).

The effect of intravenous injection of 60 gamma of adrenalin is shown in Fig. 2.<sup>§</sup> Initially there is a marked increase in velocity, due to augmented cardiac output, resulting in a 46% rise in peripheral

TABLE I.

Section of record	Avg peripheral flow, cc/sec	Avg regurgitation, cc/sec	Avg forward flow, cc/sec	Approx. time intervals, sec
Fig. 2A;00	.67	negligible		Betw. 2A and 2B: 30
" 2B;00	.98	"		" 2B and 2C: 90
" 2C;00	.07	.12	.19	" 3A and 3B: 20
" 3A;00	.36	negligible		" 3B and 3C: 60
" 3B;11	.41	"		
" 3B;22	.40	.05	.45	
" 3B;33	.50	.14	.64	
" 3B;44	.84	.04	.88	
" 3C;00	.62	negligible		

<sup>‡</sup> Identical experiments were performed with exposed vessels using a small magnet (Feitelberg, not published).

<sup>§</sup> Sections A of Fig. 2 and 3 represent normal flow before the injection of the drug. The arrows indicate the moment of injection. The time intervals between the sections of the record are listed in Table I.

blood flow (Fig. 2 B). During the following phase peripheral vasoconstriction apparently results in a marked elastic recoil of the blood, causing a diastolic regurgitation. The resultant average peripheral flow is thus reduced to 10% of the normal (Fig. 2 C). The contour of the velocity curve is obtained by joining the *upper edges* of the individual dots. The area corresponding to negative flow is, therefore, not as great as might appear at the first glance.

The intravenous injection of 25 mg of Papaverin (Fig. 3) is followed by an almost immediate rise in peripheral blood flow reaching a maximum of 230% of the normal (Fig. 3 B, Sec. 44) and persisting at an elevated level for an appreciable period of time (Fig. 3 C). Fig. 3 B shows an interesting gradual change of the diastolic contour of the flow tracing which indicates a transient diastolic regurgitation of short duration. But, as Table I shows, the average flow remains above normal even in those sections where the regurgitation is greatest.

In the beginning of record 3 B there is a noteworthy prolongation of the diastolic interval which is followed by a vigorous systolic thrust.

*Summary.* A modified electro-magnetic flow meter has been described which may be applied to investigation of a wide variety of cardiovascular problems involving flow and pressure measurements in intact animals.

## 13128

### New Media for the Growth of *Bartonella bacilliformis*.

QUENTIN M. GEIMAN. (Introduced by J. H. Mueller.)

*From the Department of Comparative Pathology and Tropical Medicine, Harvard Medical School and School of Public Health.*

Liquid, semi-solid and solid media, tissue cultures, and developing chick embryos have been utilized for the growth of *Bartonella bacilliformis*.<sup>1-5</sup> Of these media, the semi-solid leptospira medium of

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1 Noguchi, Hideyo, and Battistini, Telémaco S., *J. Exp. Med.*, 1926, **43**, 851.

2 Samper, Bernardo, and Montoya, Juan Antonio, *Rev. Fac. Medicina de Bogotá*, 1940, **9**, 197.

3 Jiménez, J. F., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **45**, 402.

4 Pinkerton, Henry, and Weinman, David, *PROC. SOC. EXP. BIOL. AND MED.*, 1937, **37**, 587.

5 Jiménez, J. F., and Buddingh, G. John, *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **45**, 546.

Noguchi and nutrient blood agar have aided (1) in proving *B. bacilliformis* as the etiological agent of Oroya fever and *verruca peruana*, the two entities of Carrion's disease in Peru, (2) animal experimentation, (3) studies on transmission, and (4) limited immunological studies with the organism. Further progress, particularly with the biochemical characteristics of the organism and the immunology of the disease, has been handicapped by the slowness and relatively sparse growth of bartonella in leptospira medium, and the difficulty of making suspensions with organisms when grown on blood agar. Thus, it was believed that the development of methods for cultivating greater quantities of the organisms would stimulate attempts to solve some of the current problems of bartonellosis.

A fruitful approach to the problem of improving known media and devising new ones, appeared to be linked with the need in leptospira medium for serum and particularly a solution of hemoglobin from laked red blood corpuscles. The necessity for these ingredients indicated that certain vitamins and growth-promoting factors found in the cellular elements of the blood might be the key substances needed for growth.\* Furthermore, *B. bacilliformis* in Oroya fever invades and multiplies within cells of the reticulo-endothelial system,<sup>4</sup> a fact which suggested the possible advantageous use of ingredients in monocyte media<sup>6, 7</sup> for enhancing the growth of bartonella.

Extensive experiments with protein digests; blood, yeast, and liver extracts; peptones; vitamins and developing chick embryos have yielded numerous new media that surpass the results obtainable with leptospira medium and blood agar. The preferred media described below are considered practical, giving growth of *B. bacilliformis* in measurable quantities.

Five strains of *B. bacilliformis* from Peru were used for the experiments and were isolated as follows: Strain 1, March 10, 1939, from a child with acute Oroya fever; Strain 2, August 3, 1939, from the proboscis of a wild fed sandfly, *Phlebotomus verrucarum*, taken at Huinco in the Santa Eulalia valley; Strain 3, August 24, 1940, from an adult during a third recurring attack of *verruca peruana*; Strain 4, August 7, 1940, from an adult in the Dos de Mayo Hospital, Lima, who had contracted Oroya fever in the Department of Ancash; Strain 5, August 7, 1940, from an adult in the Dos de Mayo Hospital, Lima, who had contracted acute Oroya fever in

\* Jiménez (1940) has recently claimed that *B. bacilliformis* requires the x factor from blood but not the v factor from yeast for growth.

<sup>6</sup> Baker, Lillian E., *Science*, 1936, **83**, 605.

<sup>7</sup> Baker, Lillian E., and Ebeling, Albert H., *J. Exp. Med.*, 1939, **69**, 365.

the Santa Eulalia valley. Strains 1, 2, and 3 were isolated by Dr. Marshall Hertig during studies on the transmission of Carrion's disease, and Strains 4 and 5 were isolated by Messrs. Eiseman, Howe, and Plimpton during preliminary immunological studies with Carrion's disease. Sincere acknowledgments are expressed for the cultures and also for the arrangements, cordial coöperation, and the provision of generous facilities for investigation by Director Telémaco Battistini, Instituto Nacional de Higiene y Salud Publica, Lima.

The liquid tryptone-serum medium is mixed as follows: 75 cc of a sterile 1% solution of tryptone<sup>†</sup> in distilled water adjusted to pH 7.6-7.8, 25 cc of fresh rabbit serum, and 0.2 cc of a sterile (Berkefeld N) mixture of ascorbic acid<sup>‡</sup>-glutathione solution (10 mg ascorbic acid and 40 mg glutathione in 20 cc Ringer's solution<sup>7</sup>). Place 8 or 10 cc of this mixture in small sterile Erlenmeyer flasks (50 cc), incubate for sterility and then inoculate with bartonella. Growth (maximum at 28°C) takes place on the bottom of the flask, becoming visible as a finely divided sediment within 24 to 48 hours and reaching a peak on about the tenth day. Depending upon the size of the original inoculum, an amount up to 0.1 cc of packed organisms can be harvested from each flask. These organisms can be re-suspended in 3-5 cc of buffered saline solution pH 7.8-8.0 to make a homogeneous suspension for use in testing immune sera for agglutinins.

The semi-solid medium is made as follows: Base agar—2 g Bacto-agar, 8.5 g sodium chloride, 1000 cc distilled water. Dissolve ingredients and adjust to pH 7.6-7.8. To 100 cc of this base medium, dissolved and cooled to 45°C, add 10 cc of sterile 5% aqueous tryptone adjusted to pH 7.6-7.8, 10 cc of fresh sterile rabbit or sheep serum, and 0.2 cc ascorbic acid-glutathione solution. Dispense in sterile test tubes, incubate, insert rubber stoppers to prevent evaporation if desired, and inoculate. At 28°C, growth becomes grossly visible at 36 to 48 hours and very heavy at about the fifth day. Minute inocula that fail to grow out on leptospira medium develop very readily on this medium. The medium is colorless and transparent, characteristics which facilitate early detection and description of growth. If tubes are kept at room temperature, the period of growth is lengthened and subcultures can be made with safety at intervals of 6 weeks to 2 months.

The solid medium is made as follows: Base agar—20 g Bacto-

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<sup>†</sup> Courtesy Difco Laboratories, Inc.

<sup>‡</sup> Courtesy Merck & Co.



agar, 20 g tryptone or proteose peptone No. 3,<sup>§</sup> 5 g sodium chloride dissolved in 1000 cc distilled water and adjusted to pH 7.6-7.8. To 75 cc of sterile and dissolved base agar cooled to 45°C, add 25 cc of fresh (rabbit or sheep) serum or, for economical reasons, 25 cc of whole defibrinated blood from rabbits or sheep, and 0.2 cc ascorbic acid-glutathione solution. Place sufficient quantities in test tubes to make a short butt and a long slant. When slants are set, place sterile rubber stoppers in the tubes and incubate for sterility. Inoculate the slant with *B. bacilliformis* by the loop method and incubate at 28°C. Growth follows the streak of the loop, becoming grossly visible within 24 to 48 hours and reaching a maximum in 10 to 14 days. Heavy growth takes place also in the water of condensation. The transparency of the medium containing serum facilitates observation of growth and general morphology of colonies. Single colonies of the 5 strains that have been tested are consistent in their morphology, being smooth, convex, mucoid, and opaque, and reaching a maximum diameter of 1-1.5 mm. However, growth is usually in a sheet, having a characteristic mucoid and opaque appearance. Since the surface of the slant must be moist to obtain maximum growth, the organisms can be harvested from the slants very easily by washing and careful scraping. Organisms grown on this medium can be used for making suspensions, although they are not as easily dispersed as those grown on the liquid medium. If defibrinated blood instead of serum is used in the medium, the quantity of growth is about the same, but the inclusion of blood cells or laked solutions of blood is not necessary for growth or maintenance of bartonella by subculture.

The media described above can be made easily and they give relatively rapid and luxuriant growth of *B. bacilliformis* when compared with results on older types of media. Thus, measurable quantities of the human bartonella can now be grown to permit bacteriological and immunological studies, the results of which hold promise of leading to methods for the control of Carrion's disease. This possibility assumes added significance when it is remembered that new foci of Carrion's disease have been reported in Colombia and Ecuador in recent years, and that no satisfactory treatment for the disease is known.

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<sup>§</sup> Courtesy Difco Laboratories, Inc.



## Site of Vitamin A Storage in the Liver.

ALVIN J. COX. (Introduced by William Dock.)

*From the Department of Pathology, Stanford University School of Medicine, San Francisco.*

While it is generally accepted that the liver is the major depot for storage of Vitamin A in the body, the exact site of deposition of this substance has not been clearly defined. After administration of large doses of Vitamin A to animals, accumulation of a stainable lipoid in the Kupffer cells has been reported.<sup>1, 2</sup> However, others have seen little change in the Kupffer cells, and one report<sup>3</sup> describes lipoid deposition in the hepatic cells under these conditions. Popper's studies with the fluorescence microscope<sup>4</sup> suggest that Vitamin A is stored in both hepatic and Kupffer cells. In some cases of cirrhosis of the liver the Vitamin A stores are much depleted, and it has been pointed out<sup>5</sup> that this depletion is most marked in cases where active degeneration of liver cells is present, although histological evidence of Kupffer cell damage has not been noted. Lasch<sup>6, 7</sup> has provided the best evidence that the storage occurs in the Kupffer cells. He found decreased storage of administered Vitamin A in the livers of rabbit and guinea pigs after blockading the reticulo-endothelial cells with a mixture of thorotrast, trypan red, trypan blue, and ferric oxide. He noted further that severe poisoning with phosphorus, which damaged the hepatic cells, caused no decrease in the ability of the liver to store this vitamin.

A study of 3 human cases which had passed through an episode of massive necrosis of the liver has yielded evidence that the hepatic parenchymal cells are not essential for Vitamin A storage. In each of these cases large amounts of liver substance had been converted to vascular connective tissue in which there were many small bile ducts but no demonstrable hepatic cells in histological sections. Each liver contained large, quite sharply demarcated nodules of normally arranged liver tissue with moderate enlargement of the hepatic cells

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<sup>1</sup> Domagk, G., and Dobeneck, P., *Virch. Arch.*, 1935, **290**, 385.

<sup>2</sup> Strauss, K., *Beitr. z. path. Anat., u. z. allg. Path.*, 1934, **94**, 345.

<sup>3</sup> v. Drigalski, W., and Laubmann, W., *Kl. Wchnschr.*, 1933, **12**, 1171.

<sup>4</sup> Popper, H., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 133.

<sup>5</sup> Cox, A., *Am. J. Path.*, 1939, **15**, 647.

<sup>6</sup> Lasch, F., *Kl. Wchnschr.*, 1935, **14**, 1070.

<sup>7</sup> Lasch, F., and Roller, D., *Kl. Wchnschr.*, 1936, **15**, 1636.

TABLE I.  
Vitamin A Content of Liver.

	Interval following initial jaundice	Altered liver tissue	Intact liver tissue
Case 1 (7C-631)	3 weeks	+++	++
Case 2 (8C-527)	3 "	+++	++
Case 3 (9C-415)	6 mo.	++++	++

which showed no sign of injury. The appearance in each case indicated that the injurious agent had ceased acting before the patient died.

It was possible grossly to select portions of each liver which were made up largely of tissue free from hepatic cells, and to compare the Vitamin A content with that of nodules containing liver cells from the same liver. The Vitamin A determinations were made by using the Carr-Price antimony trichloride reaction on chloroform extracts of weighed samples of fresh liver dehydrated by grinding with anhydrous sodium sulphate. The blue color which developed was compared with permanent color standards which had been calibrated against a Vitamin A solution of known strength.

The quantity of Vitamin A recorded as +++ is estimated at about 250 international units per gram of liver, approximately the average amount in livers obtained at autopsy. ++ indicates half of this amount and ++++ refers to twice this value.

Although rough, these measurements show conclusively that in liver tissue from which the hepatic cells had been removed by previous massive injury (in one case the disease was related to cinchophen administration) the Vitamin A store was greater than in the portion containing hepatic cells, and therefore must have been stored elsewhere than in these cells. It is difficult to identify Kupffer cells in histological sections of the abnormal liver tissue because of the lack of any relationship to hepatic cell cords, and frequent absence of any other specific characteristics. However, in such regions there are scattered large spindle-shaped cells which contain fine fatty granules and particles of brown iron containing pigment. These are presumably Kupffer cells, and it is probable that the Vitamin A in the livers was stored in such cells. This conclusion implies that agents which produce massive liver necrosis may cause relatively little injury to Kupffer cells.

The greater concentration of Vitamin A in the abnormal regions than in the intact liver tissue may be due to concentration of Kupffer cells in the injured areas from which hepatic cells had been removed, and to hypertrophy and hyperplasia of the hepatic cells in the un-

damaged portions, thus separating the Kupffer cells and decreasing their number per unit weight of tissue.

The character of the injury in portal cirrhosis of the liver is quite different from that which has been described above, and it is possible that accompanying injury to the Kupffer cells results in the very low Vitamin A stores in this disease. However, the cause may be an insufficient supply of the vitamin, either because of impaired conversion of carotin in injured liver cells or inadequate intake of Vitamin A in patients with this disease.

*Summary.* In 3 cases of healed massive necrosis of the liver Vitamin A was more abundant in the parts of the liver from which hepatic cells had disappeared than in those where these cells had not been destroyed. Presumably it was stored in the Kupffer cells, which had been spared by the agent which produced the necrosis.

### 13130 P

#### Trypanocidal Action of 3-Nitrobenzoic Acid and Some Derivatives.

SANFORD M. ROSENTHAL AND HUGO BAUER. (Introduced by M. I. Smith.)

*From the Division of Chemotherapy, National Institute of Health, Washington, D.C.*

Various sulfonamide derivatives prepared in this laboratory have been tested against *Trypanosome equiperdum* with negative results. 4-Nitrobenzoic acid which has a slight antibacterial action<sup>1, 2</sup> was found to have a trace of trypanocidal activity. Investigation of the isomers revealed that the meta derivative showed increased activity against trypanosomes, although without effect upon bacteria; 2-nitrobenzoic was inactive against both types of infection. A review of the literature revealed that Morgenroth found 3-hydroxybenzoic acid to possess some trypanocidal action.<sup>3</sup>

A more detailed study of 3-nitrobenzoic acid and some derivatives was undertaken. Mice were inoculated intraperitoneally with a quantity of trypanosomes that would cause death in 3 to 5 days. Therapy was usually begun on the second day after inoculation. The dosage

<sup>1</sup> Mayer, R. L., and Oechslin, C., *Compt. rend. Soc. Biol.*, 1939, **130**, 211.

<sup>2</sup> Rosenthal, S. M., and Bauer, H., *U. S. Pub. Health Rep.*, 1939, **54**, 1317.

<sup>3</sup> Morgenroth, J., and Rosenthal, F., *Berlin. Klin. Wschnschr.*, 1912, **49**, 134.

used was in most cases one-half of the maximum tolerated single dose; this was repeated daily for 3 to 6 days to surviving animals. A few of the compounds were synthesized by one of us (H.B.). The others were obtained from Eastman Kodak Company.

The average survival time was calculated from the day of inoculation, using a maximum of 30 days.

Compounds with some activity are shown in Table I. The following derivatives also showed traces of activity; dosages in grams per kilo, administered subcutaneously, are shown in parenthesis. The soluble sodium salts of the acids were employed. Insoluble compounds were injected in olive oil. Dosages represent approximately one-half the single M.T.D.

3-Nitrobenzyl chloride (0.5), 3-nitrohippuric acid (2.0), 5-nitrosalicylic acid (0.5), and 3,5-dinitrosalicylic acid (0.1).

The following compounds were inactive:

2-Nitrobenzoic acid (0.5), 3-nitrophenol (0.25), 3-nitrochlorobenzene (0.25), 3-nitrobenzene sulfonamide (0.25), 3-nitrobenzene sulfonic acid (1.0), 3-nitrobenzoyl-2-amino-pyridine (0.4), 3-nitroanisole (0.5), 3-nitrophenetole (0.5), 3-nitroacetophenone (0.5), 3-aminobenzoic acid (2.0), 3-aminobenzene sulfonamide (1.5), nicotinamide (1.0), isophthalic acid (1.5), 3-nitrophthalic acid (2.0), 4-nitrophthalic acid (2.0), 3-nitrophthalimide (1.0), 3-nitrosalicylic acid (0.5), 3,5-dinitrobenzoic acid (0.25), 2,4-dinitrobenzoic acid (0.5), 3-nitro-4-hydroxy-toluene (0.5), 3-nitro-2-

TABLE I.

Trypanocidal Action of 3-Nitrobenzoic Acid and Some Related Compounds. Doses in most cases represent one-half the maximum tolerated single dose.

Compound	Route	g/kilo x days	No. of mice	Avg survival (days)
3-Nitrobenzoate sodium	s.c.	.5 x 6	60	13.3*
" " "	oral	.5 x 6	10	5.1
3-Nitrobenzoic acid (in oil)	s.c.	.5 x 4	30	6.8*
3-Nitrobenzyl alcohol " "	s.c.	.5 x 6	10	5.8
3-Nitrobenzal chloride " "	s.c.	.5 x 2	10	3.9
" " " " " "	s.c.	1.0 x 5	9	5.7
3-Nitrotoluene " "	s.c.	.25 x 3	10	3.6
3-Nitrobenzoate methyl " "	s.c.	.5 x 4	10	8.2
3-Nitrobenzoate ethyl " "	s.c.	.5 x 4	10	6.2
3-Nitrobenzaldehyde " "	s.c.	.5 x 3	10	3.5
3-Hydroxybenzoate sodium	s.c.	.5 x 3	10	3.0
" " " " " "	s.c.	1.5 x 4	10	5.5
3-Brombenzoate " "	s.c.	.25 x 3	10	5.1
Nicotinic acid " "	s.c.	1.0 x 3	10	4.0
4-Nitrobenzoate " "	s.c.	.5 x 6	10	4.9
Controls			125	3.1*

\*Average of several experiments.

aminotoluene (0.25), 3-nitro-4-aminotoluene (0.5), 3-nitro-2-iodotoluene (0.4), 3-nitro-4-amino-anisole (0.5), 3-nitro-4-aminophenetole (0.75), 3-nitro-4-acetylaminophenyl acetate (0.5), 3-nitrodiphenylene oxide (0.5).

The blood of most animals was cleared of parasites at the termination of therapy with subcutaneous injections of 3-nitrobenzoate, but the incidence of relapses was high and only 16% of the series remained parasite free. None of the derivatives reported herein proved of greater activity.

That the nitro group is not essential is shown in that some activity was retained by 3-hydroxy- and 3-brom-benzoic acid and by nicotinic acid.

The importance of the carboxy group is shown in that only those derivatives were active which contained this group or a closely related radical which could be converted into it. Replacement by OH or substituted OH groups, by Cl,  $\text{SO}_3\text{H}$  or  $\text{SO}_2\text{NH}_2$  abolished activity.

3-Nitrobenzoate shows some action against *trypanosome equiperdum in vitro*. Concentrations up to 0.1% bring about marked swelling of the organisms with decrease in motility after exposure of one hour at room temperature. The ortho isomer was without effect.

Certain differences between bacterial and trypanosome chemotherapy were noted. While sulfanilamide and 4-nitrobenzoate are more active against streptococci by mouth than by subcutaneous injection, this relationship was reversed with 3-nitrobenzoate upon trypanosomes. While 4-aminobenzoic acid antagonizes the antibacterial action of the former compounds, 3-aminobenzoic acid and nicotinamide did not antagonize 3-nitrobenzoic acid.

In view of the relationship of 4-nitrobenzoic acid to sulfanilamide in bacterial chemotherapy, the possibility of obtaining more active trypanocidal agents related to 3-nitrobenzoic acid deserves further study.



## 13131 P

**I. Acute Hypoglycemia in Newly Born Pigs.  
(So-called Baby Pig Disease).**

ROBERT GRAHAM, JESSE SAMPSON AND H. R. HESTER. (Introduced by F. W. Tanner.)

*From the Division of Animal Pathology and Hygiene, University of Illinois.*

During the past 8 years sporadic outbreaks of an unidentified, highly fatal malady of newly-born pigs have come to the attention of the Illinois Agricultural Experiment Station. This, or an indistinguishable malady, has been arbitrarily referred to as baby pig disease.\* In connection with different outbreaks on widely separated farms an increasing number of naturally affected pigs has been delivered to the laboratory for examination, while a limited number of affected herds has been inspected to observe methods of managing the pregnant sows. So far as could be determined the rations fed the pregnant sows appeared adequate. However, further information regarding the relation of feeding of the pregnant sow to the disease in newly-born litters is desirable.

Pigs that suffer from the disease referred to present a characteristic syndrome. In typical outbreaks newly-born pigs, though apparently normal at birth, suddenly develop symptoms. Pigs that immediately display similar or indistinguishable symptoms at birth are purposely excluded from consideration in this report. In the typical syndrome, apparently normal litters at approximately 24 to 48 hours of age show symptoms of shivering, dullness and inappetence. Affected pigs often emit a weak crying squeal. Coincident with the loss of appetite and weakness, the hair coat becomes rough and the affected pigs leave the nest and lapse into coma. Death of several or all pigs in the affected litters often occurs within 24 to 36 hours after the first symptoms are manifested. The extent of the loss has ranged from one to 22 litters, representing approximately 5 to 95% of the pigs farrowed in some herds.

At autopsy no gross pathologic lesions have been observed. Supplementing gross autopsy examination of typically affected pigs repeated efforts have been made to demonstrate in the tissues the presence of pathogenic agents such as bacteria, filtrable viruses, protozoa and toxins. The results proved negative. Failure to reproduce the disease experimentally or to demonstrate the presence of an in-

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\* Mimeographed release (Rev. 1940), College of Agriculture, Extension Service.

fectious agent prompted a chemical examination of the blood. Analyses for calcium, inorganic phosphorus, and ketone bodies revealed values within the normal range for these constituents. However, abnormal amounts of blood sugar have been consistently encountered.<sup>†</sup> The sugar level ranged from approximately 3 to 61 mg per 100 cc. In one series of 20 affected pigs from 7 different litters, an average of 26 mg of sugar per 100 cc of blood was found. The blood sugar level in normal pigs of the same age ranged from 99 to 131 mg per 100 cc with an average of 113 mg.

A possible prenatal influence on this pathologic condition in newly-born pigs is obviously suggested; but until the underlying cause or causes are established, the cryptogenic nature of the acute hypoglycemia is recognized. Contributory evidence in support of the possible primary significance of acute hypoglycemia has been observed in the therapeutic response in naturally affected pigs following repeated injections of glucose solution. Pigs in the early stage of the disease show improvement in 2 to 3 hours following dextrose therapy, while repeated injections of dextrose together with forced feeding of milk have demonstrated that the life of naturally affected pigs may be prolonged and that in some cases the treated pigs may recover. However, glucose therapy even if repeated appears ineffective in the terminal stages of the disease.

### 13132

#### Influence of Sucrose upon Production of Serologically Reactive Material by Certain Streptococci.

JAMES M. NEILL, JOHN Y. SUGG, EDWARD J. HEHRE AND  
EVELYN JAFFE.

*From the Department of Bacteriology and Immunology, Cornell University  
Medical College, New York.\**

This paper deals with the potential capacity of certain streptococci from man to produce material reactive with types 2 and 20 anti-pneumococcus and with antileuconostoc serums. The point of major interest is the influence of sucrose upon the production of the reactive material.

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<sup>†</sup> The blood sugar was determined by the Shaffer-Hartmann-Somogyi method according to the technic of Koch. (Koch, F. C., *Practical Methods in Biochemistry*, Williams and Wilkins, Baltimore, 1934.)

\* Aid was received by a grant from the Ruth B. Ettinger Fund.

It has long been known that some bacteria, especially *Leuconostoc mesenteroides*, produce abundant amounts of polysaccharide when grown in media containing sucrose but produce little or none when other common carbohydrates are substituted for sucrose. We<sup>1</sup> have found that the leuconostoc polysaccharides (dextrans) are reactive not only with antisera of leuconostoc but also with antisera of types 2 and 20 (and sometimes also of type 12) pneumococci. This newer evidence showed that the influence of sucrose upon the formation of the leuconostoc dextrans actually represented an example of the influence of a particular carbohydrate constituent of the culture medium upon the capacity of a microorganism to elaborate a serologically reactive polysaccharide. A study was then made to determine if the same or a similar phenomenon occurred among other sucrose-fermenting bacteria particularly among streptococci.

That some streptococci from man produce large amounts of "slime" or gum-like material when grown on sucrose media is an old observation. Hlava<sup>2</sup> isolated streptococci from patients with scarlet fever and other infections which were so similar to the common leuconostoc from plants in respect to mucoid colonies on sucrose and non-mucoid colonies on glucose media that he called them *Leuconostoc hominis*. Later Oerskov and associates<sup>3</sup> found that normal throats also had streptococci that produced large amounts of gum from sucrose or raffinose but not from other common carbohydrates. Recently Sherman and his associates<sup>4</sup> have reported that some members of the *Streptococcus salivarius* group possess the property of forming mucoid colonies on sucrose but not on glucose agar; their data on the serological properties of the polysaccharide formed by those streptococci have not yet been published. In addition to these reports there probably have been a number of unreported observations of the influence of sucrose upon the mucoid appearance of streptococcus cultures.

For the present study a collection of streptococci principally of human origin and a number of other sucrose-fermenting bacteria were tested. The streptococcus strains consisted of 30 group A, 5 B, 3 C, 8 D, 2 E, 5 F, 10 G, 8 H, 7 K, 2 "indifferent" strains which would be classified as *Strep. salivarius*, and 3 strains of *Strep. lactis* which were obtained through the courtesy of Dr. Sherman; most of

<sup>1</sup> Sugg, J. Y., and Hehre, E. J., in press.

<sup>2</sup> Hlava, J., *C. Bakt.*, 1902, **32**, 263.

<sup>3</sup> Oerskov, J., and Poulsen, K. A., *Z. Bakt. I. Orig.*, 1931, **120**, 125; Koch, F. E., *Z. Bakt. I. Orig.*, 1934, **130**, 381.

<sup>4</sup> Niven, C. F., Smiley, K. L., and Sherman, J. M., *J. Bact.*, 1941, **41**, 479.

the other streptococci were kindly supplied by Dr. Lancefield; all except 4 of group *D* fermented sucrose with acid production. The other bacteria tested were 5 strains of lactobacilli from plants, 5 of Friedlander *A*, *B*, and *C*; and one each of aerogenes (*A* 11), coli communior, proteus, and a spore-forming bacillus. For comparison with the streptococci the data on the serological properties of a collection of 28 strains of leuconostoc are included. These strains had been isolated from various plants obtained from diverse geographical sources; their mutual properties of fermenting sucrose and pentoses might justify the term *Leuconostoc mesenteroides*<sup>5</sup> for all of them. The chemical and serological properties of the leuconostoc dextrans have been described in another paper.<sup>1</sup> (In view of the serological relationship which will be shown to exist between them it should be noted that the leuconostoc strains can be clearly differentiated from the group *H* streptococci on the basis of other properties, among which are the failure of any of the group *H* to ferment arabinose or xylose and the higher optimum temperature for growth of the streptococci.)

Equal amounts of each of the strains were inoculated into 2 tubes of medium consisting of 2% Neopeptone, 0.5% sodium chloride, and 0.2% anhydrous disodium phosphate plus 1% dextrose or 1% sucrose. After 5 days of incubation the cultures were centrifuged and the supernatant fluids after being neutralized and heated at 70°C for 30 minutes, were used as antigens. Each antigen was tested in 1:10 and 1:100 dilutions against 1:10 dilutions of types 2, 20, 12 and 1 antipneumococcus serums and against the antisera of two strains of *Leuconostoc mesenteroides* which are designated *A* and *B* in the protocol; the fluids which reacted in the 1:100 dilution were tested in 1:1,000 and 1:10,000 dilution. The pneumococcus serums had been produced by immunization of rabbits with bacteria grown in meat infusion peptone broth without added carbohydrate; the leuconostoc serums with bacteria grown in media containing sucrose. The results are summarized in Table I.

It is evident (Table I) that when grown in sucrose broth all of the leuconostoc and 7 of the 8 group *H* streptococci produced large amounts of material which was reactive with the antisera of types 2 and 20 pneumococci and of the two strains of leuconostoc; the material produced by 4 of the leuconostoc strains had the additional property of reacting with type 12. In contrast in the culture mediums in which dextrose was substituted for sucrose, none of the

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<sup>5</sup> Hucker, G. J., and Pederson, C. S., in *Bergey's Manual of Determinative Bacteriology*, Baltimore, 1939, p. 359.



leuconostoc or group *H* streptococci produced detectable amounts of the reactive material. That the capacity of elaborating it from sucrose is not as frequent among all groups of streptococci as among the group *H* variety is suggested by the negative results obtained with the strains of other groups which were tested. A more comprehensive collection will be studied in order to get better information on its occurrence among streptococci.

The group *H* strains which reacted had been isolated from throats; six were originally from Dr. Hare's<sup>6</sup> collection; one had been isolated by Dr. Julia M. Coffey in the laboratories of the New York State Department of Health. All of these strains were as non-reactive when grown in maltose or lactose broth as when grown in dextrose broth.

In order to distinguish between the antigen involved in the usual Lancefield grouping test and the antigens involved in the described reactions of the sucrose cultures of group *H* streptococci the following experiment was made. Two of the strains of group *H* streptococci which had reacted with the pneumococcus and leuconostoc antisera were grown in meat infusion peptone broth containing 1% dextrose and in the same medium containing 1% sucrose. The 18-hour-old cultures were centrifuged at high speed and the supernatant fluids removed as completely as possible from the sedimented bacteria. The bacterial cells were then washed with physiological salt solution and recentrifuged. An HCl-extract of the washed cells was then prepared by the usual Lancefield<sup>7</sup> procedure. The supernatant fluids and the HCl-extract of the washed cells obtained from the dextrose and the sucrose cultures of the two strains were tested in a series of dilutions against 1:10 dilutions of the following rabbit antisera: Group *H* streptococcus (which was kindly supplied by Dr. Lancefield), leuconostoc (*A* and *B*) and types 2, 20, 12 and 1 pneumococcus; the streptococci used for the immunization had been grown in broth containing dextrose, the leuconostoc in broth or agar containing sucrose and the pneumococci in broth containing no added carbohydrate. The results are in Table II.

The chief point in the results (Table II) was that when tested against the streptococcus *H* antiserum both the supernatant fluids and the HCl-extracts obtained from the dextrose both cultures reacted in as high dilution as did the corresponding materials obtained from the sucrose broth cultures. This point indicates that the antigens reactive with the group *H* antiserum are different from

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<sup>6</sup> Hare, R., *J. Path. Bact.*, 1935, **41**, 499.

<sup>7</sup> Lancefield, R. C., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **38**, 473.



TABLE I.  
Serological Reactions of the Supernatant Fluids of Sucrose and of Dextrose Broth Cultures.

Kind of bacteria	No. of strains	Sucrose cultures <i>vs.</i> the antisera					Dextrose cultures <i>vs.</i> the same antisera		
		Antipneumococcus				Antileuconostoc			
		2	20	12	1			A	B
<i>Leuconostoc mesenteroides</i> (28)	4	+	+	+	+	+	+	+	0
	24	+	+	+	+	+	+	+	0
<i>Streptococcus</i> Lancefield H (8)	7	+	+	+	+	+	+	+	0
	1	0	0	0	0	0	0	0	0
<i>Streptococcus A</i> (30), <i>B</i> (5), <i>C</i> (3), <i>D</i> (8), <i>E</i> (2), <i>F</i> (5), <i>G</i> (10), <i>K</i> (7)	70	0	0	0	0	0	0	0	0
<i>Strep. salivarius</i> (2), <i>lactis</i> (3)	5	0	0	0	0	0	0	0	0
Other bacteria	14	0	0	0	0	0	0	0	0

0, no precipitation with 1:10 or higher dilutions of the antigens.

++, precipitation with 1:1,000 and in some instances 1:10,000 dilutions of the antigens.

TABLE II.

Comparison of Reactions of *Streptococcus H* "grouping," Serum with the Reactions of Types 2 and 20 Antipneumococcus and of Antileuconostoc Serums.

Antisera	Supernatant fluids of cultures of group <i>H</i> streptococci						HCl-extract of group <i>H</i> streptococcus cells			
	Sucrose cultures		Dextrose cultures		Dextrose cultures		Sucrose cultures		Dextrose cultures	
	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2
<i>Streptococcus H</i> (strain 1)	++	+	++	+	++	++	++	++	++	++
<i>Leuconostoc</i> (strain A or B)	++++	++++	0	0	+++	+++	+++	+++	0	0
<i>Pneumococcus</i> type 2 or 20	0	0	0	0	0	0	0	0	0	0
" " 1 or 12										

0 = no precipitation with undiluted or diluted antigen.

+, ++, +++, ++++ = precipitation with undiluted, 1:10, 1:100 and 1:1,000 dilutions of the antigens.

those involved in the reactions of the pneumococcus and leuconostoc antisera.

Another item of interest is the comparative amounts of the material reactive with the different sera which are contained in the supernatant fluids of the sucrose broth cultures. For example, the supernatant fluids of the 7 strains of the group *H* streptococci grown in that medium invariably reacted in 1:1,000 and often in 1:10,000 dilution against the pneumococcus and leuconostoc sera whereas in experiments made against group *H* antisera none of the same fluids reacted in 1:100 dilution and the majority failed to react in 1:10 dilution.

A polysaccharide has been prepared from sucrose broth culture of one of the group *H* streptococci and although its study is not completed it has been proved to be a dextro-rotatory substance. Further investigation of the relationships of this polysaccharide to the leuconostoc dextrans<sup>1</sup> has been delayed until we obtain potent antisera by immunization with group *H* streptococci grown in sucrose media.

*Summary.* When grown in sucrose broth some strains of group *H* streptococci produced large amounts of material reactive with types 2 and 20 antipneumococcus and with antileuconostoc sera; little or none was produced by the same streptococci when grown in dextrose broth. This reactive material was different from the streptococcus antigen involved in the usual Lancefield grouping test; as much of the latter was produced in dextrose as in sucrose broth culture.

In addition to indicating an interrelationship of the different Gram-positive cocci the data furnished an example of the influence of a particular carbohydrate upon the capacity of some microorganisms to elaborate a serologically reactive polysaccharide.

### 13133

#### A Selective Medium for the Isolation of *Streptococcus salivarius*.

KENNETH D. ROSE AND CARL E. GEORGI. (Introduced by  
H. G. O. Holck.)

*From the Department of Bacteriology, University of Nebraska, Lincoln.*

Restaurant eating utensils have long been recognized as potential vectors in the transmission of respiratory and related diseases. Extensive investigations have been reported by many workers con-

cerning technics applicable to sanitary surveys of restaurants. Their results have consistently demonstrated the appearance of Gram-negative bacilli and Gram-positive rods and micrococci on washed glasses. Recent trends have been directed toward the use of an organism, normal to the human mouth, as an index for the study of oral pollution. For the detection of a specific bacterium in a mixed population occurring on washed glasses, an inhibitory medium is desirable if not essential. These investigations concern the development of a selective medium for the isolation of a specific oral streptococcus, the presence of which on drinking glasses may be used as an index of salivary contamination.

Fleming and Young<sup>1</sup> and Bornstein,<sup>2</sup> utilizing the inhibitory powers of potassium tellurite on Gram-negative bacteria, employed this salt in media used for the isolation of fecal streptococci. Dick and Hucker<sup>3</sup> demonstrated that a 1:400,000 concentration of crystal violet inhibited Gram-positive rods and micrococci in enrichment broths intended for primary isolations of streptococci from washed glasses. Niven, *et al.*,<sup>4</sup> reported mucoid colony formation by certain "typical" strains of *Streptococcus salivarius*, characterized by Safford, *et al.*,<sup>5</sup> when cultured on a substrate containing sucrose. Combining these 3 factors, a medium was developed which would effectively eliminate Gram-negative bacteria and Gram-positive rods and micrococci from mixed cultures, but permitting "typical" *Streptococcus salivarius* to grow with a characteristic gummy or mucoid colony.

*Methods and Results.* Twenty species of Gram-negative and Gram-positive organisms and 14 strains of *Streptococcus salivarius* were employed in these experiments. Plates of the basal substrate containing varying amounts of the inhibitory substances were streaked with 24-hour-old broth cultures and incubated for 48 hours at 37°C prior to observation. The concentrations of the inhibitory agents arrived at and yielding the more promising results were determined as 0.03% potassium tellurite and 1:500,000 crystal violet. These concentrations agree favorably with those used by Garrod.<sup>6</sup> The basic substrate used was a modification of that suggested by Safford, *et al.*,<sup>5</sup> and is as follows:

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<sup>1</sup> Fleming, A., and Young, M. Y., *J. Path. and Bact.*, 1940, **51**, 29.

<sup>2</sup> Bornstein, S., *J. Bact.*, 1940, **39**, 383.

<sup>3</sup> Dick, L. A., and Hucker, G. J., *J. Milk Tech.*, 1940, **3**, 307.

<sup>4</sup> Niven, F., Jr., Smiley, K. L., and Sherman, J. M., *J. Bact.*, 1941, **41**, 479.

<sup>5</sup> Safford, C. E., Sherman, J. M., and Hodge, H. M., *J. Bact.*, 1937, **33**, 263.

<sup>6</sup> Garrod, P., *St. Barth. Hosp. Rep.*, 1933, **66**, 203.

Proteose peptone (Difeo)	5.0 g
Beef extract (Bacto)	3.0 "
Yeast extract (Bacto)	5.0 "
Glucose	1.0 "
Sucrose	10.0 "
K <sub>2</sub> TeO <sub>3</sub> (1%)	30.0 ml
Crystal violet (1%)	0.2 "
Agar (Bacto)	15.0 g
Distilled water to make	1000.0 ml
Adjust to pH 7.4	

Aqueous solutions of potassium tellurite and crystal violet were added aseptically after the basal medium had been autoclaved at 15 lb for 15 minutes. The effect of potassium tellurite, crystal violet and sucrose upon the growth of test organisms and on gum formation by *Streptococcus salivarius* is shown in Table I.

The colony formation is somewhat different from that described

TABLE I.  
Effect of 0.03% Potassium Tellurite and 1:500,000 Crystal Violet on 21 Bacterial Species.

Bacterial species	No. of strains	Medium		
		A	B	C
<i>Streptococcus salivarius</i> (typical strains)*	11	++†	++†	++†
<i>Streptococcus salivarius</i> (atypical strains)*	3	++	++	++
<i>Streptococcus</i> species (from chicken feces)	3	++	++	—
<i>Streptococcus lactis</i>	1	++	++	++
<i>Streptococcus liquefaciens</i>	1	++	++	++
<i>Streptococcus pyogenes</i>	1	++	++	++
<i>Streptococcus equi</i>	1	++	++	++
<i>Streptococcus agalactiae</i>	2	++	±	—
<i>Bacillus subtilis</i>	1	++	++	—
<i>Bacillus mycoides</i>	1	—	—	—
<i>Bacillus megatherium</i>	1	—	—	—
<i>Staphylococcus aureus</i>	1	++	+	—
<i>Staphylococcus albus</i>	1	++	—	—
<i>Gaffkya tetragens</i>	1	++	—	—
<i>Lactobacillus delbrückii</i>	1	—	—	—
<i>Mycobacterium lacticola</i>	1	+	+	+
<i>Escherichia coli</i>	1	—	—	—
<i>Aerobacter aerogenes</i>	1	—	—	—
<i>Eberthella typhosa</i> (Hopkins)	1	—	—	—
<i>Shigella dysenteriae</i>	1	—	—	—
<i>Salmonella schottmuellerii</i>	1	—	—	—
<i>Neisseria catarrhalis</i>	1	—	—	—

\*One atypical and 2 typical strains obtained from Dr. J. M. Sherman, Cornell University.

— No growth.

+ Growth.

++ Good growth.

† Gum formation.

Medium A = Basal substrate without inhibitory agents.

" B = " " + 0.03% potassium tellurite.

" C = " " + " " " " and 1:500,000 crystal violet.

by Niven, *et al.*<sup>4</sup> *Streptococcus salivarius* produces surface colonies 4 to 5 mm in diameter and hemispherical in shape. Potassium tellurite is generally precipitated as metallic tellurium at the periphery of the base of the colony while the crystal violet appears as a bluish halo in the medium directly surrounding the colony.

Before any practical application of this medium could be made, the distribution of the gum-forming "typical" *Streptococcus salivarius* had to be determined. Swabbings from human lips, washed and unwashed glasses, floor dust, storage trays and drain boards were obtained using the technic of Winslow and Sanjiyan,<sup>7</sup> modified by employing rolled cotton swabs instead of flannel-covered discs. Inocula from enrichment cultures of tap, wash and rinse waters were streaked on the selective medium. Restaurant air samples were obtained by exposing plates of the selective medium for 15 minutes. All plates were incubated at 37°C for 48 hours and then examined for the characteristic gummy colony of *Streptococcus salivarius*. The results of these distribution studies appear in Table II. Six of 50 strains of gum-forming streptococci isolated were studied critically. Their biochemical and cultural characteristics were found to be similar to the "typical" *Streptococcus salivarius* described by Safford, *et al.*<sup>5</sup>

*Conclusions.* A medium has been developed on which a specific oral streptococcus can be selectively isolated from mixed bacterial populations. The value of this selective medium as a detector of oral contamination of restaurant eating utensils is indicated by the distribution studies, which show that this bacterium is found normally in the human mouth and on articles which have come in direct contact with human lips.

TABLE II.  
Recovery of "Typical" *Streptococcus salivarius* from Various Sources.

Source	Samples No.	<i>S. salivarius</i> No.	<i>S. salivarius</i> %
Human lips	45	42	93.4
Unwashed glasses	40	18	45.0
Washed glasses	40	4	10.0
Drain boards	6	1	16.6
Storage trays	9	0	0.0
Dust (Washroom)	12	2	16.6
Dust (Service area)	12	1	8.3
Tap water	5	0	0.0
Wash water	5	0	0.0
Rinse water	5	0	0.0
Air (Washroom)	3	0	0.0
Air (Service area)	6	0	0.0

<sup>7</sup> Winslow, C.-E. A., and Sanjiyan, D. H., *J. Bact.*, 1924, **9**, 559.



## Neutralization of Angiotonin by Normal and by Ischemic Kidney Blood Plasma.

MEYER FRIEDMAN.

*From the Harold Brunn Institute for Cardio-Vascular Research, Mount Zion Hospital, San Francisco, Calif.*

The blood<sup>1</sup> leaving the partially ischemic kidney has been found to differ from normal systemic blood in that it contains a pressor substance similar to angiotonin<sup>2</sup> in its physiological action, but dissimilar in its chemical properties.

In the present communication evidence is presented which indicates that blood leaving the hemodynamically altered kidney also differs from normal blood in its comparative inability to neutralize the pressor action of angiotonin.

*Methods.* In each of 6 experiments a dog was anesthetized with pentobarbital sodium. Sufficient arterial blood was then obtained by femoral puncture for 3 samples (5 cc each) of plasma. The left kidney of each dog was exposed, the left renal artery partially clamped, and 75-100 cc of renal venous blood were collected as described in a previous report.<sup>1</sup> Purified heparin (Connaught) was used as the anti-coagulant. Five samples (5 cc each) of plasma were obtained from this renal blood collection. Thus, from each dog 3 samples of normal blood plasma and 5 samples of ischemic kidney plasma were obtained. Angiotonin (0.2 to 0.8 cc) was added to each of the 3 normal blood plasma samples and to 3 of the 5 renal plasma samples. All samples, including the 2 remaining renal plasma samples, were incubated at 38°C for one hour. In each experiment, angiotonin alone, equal in quantity to the amount added to the plasma samples, was also incubated.

The various samples, after incubation, were injected in the femoral vein of an anesthetized, previously nephrectomized dog (24 hours) and the pressure changes continuously followed by means of a mercury manometer connected by cannula to the femoral artery.

*Results.* As Table I and Fig. 1 indicate, there is practically complete neutralization of the angiotonin by the normal systemic blood plasma after an hour's incubation. The average pressure rise following the injection of the normal blood plasma-angiotonin mixtures was 5 mm of Hg, and only in Experiment VI was there a sig-

<sup>1</sup> Friedman, M., Selzer, A., and Sampson, J., *Am. J. Physiol.*, 1941, **131**, 799.

<sup>2</sup> Page, I. H., and Helmer, O. M., *J. Exp. Med.*, 1940, **71**, 29.

nificant rise following its injection. However, it was found that ischemic kidney plasma lacked the property of angiotonin neutralization possessed by normal systemic blood plasma, for the average rise following the injection of ischemic kidney blood plasma-angiotonin mixture was 20 mm of Hg. This latter rise was comparable to the pressor effect obtained following the injection of angiotonin in saline alone. The pressor rise following the injection of the ischemic kidney plasma-angiotonin mixture was not due to the pressor property of ischemic kidney blood for, as can be seen by an examination of the data, the rise following injection of 5 cc of incubated ischemic kidney blood plasma was negligible.

*Discussion.* It has been recognized<sup>3</sup> that angiotonin and its counterpart, hypertensin,<sup>4</sup> may be inhibited by normal blood plasma.

TABLE I.

Effect of Ischemic Kidney Blood Plasma and Normal Blood Plasma on Angiotonin.

Exp. No.	Amt of angiotonin (cc)	Blood pressure effect of angiotonin alone (mm of Hg)	Blood pressure effect of angiotonin plus ischemic kidney plasma* (mm of Hg)	Blood pressure effect of angiotonin plus normal plasma† (mm of Hg)	Blood pressure rise of ischemic kidney plasma alone‡ (mm of Hg)
I-a	0.2	+30	+15	0	+8
-b	0.2		+20	0	+4
-c	0.2		+20	0	
II-a	0.2	+20	+20	0	+3
-b	0.2		+15	0	+5
-c	0.2		+15	+ 5	
III-a	0.3	+23	+20	+ 3	+2
-b	0.3		+15	+12	+3
-c	0.3		+18		
IV-a	0.5	+27	+20	0	+5
-b	0.5		+19	+ 2	+6
-c	0.5			+ 2	
V-a	0.5	+20	+17	+10	+2
-b	0.5		+13	+ 5	0
-c	0.5		+17		
VI-a	0.8	+35	+32	+15	+4
-b	0.8		+32	+15	0
-c	0.8		+32	+12	
Avg	.41	+26.0	+20.0	+ 5.0	+3.5

\*Five cc of ischemic kidney plasma were used in each experiment.

†Five cc of normal plasma were used in each experiment.

‡Five cc of incubated ischemic kidney plasma injected in each experiment.

<sup>3</sup> Page, I. H., and Helmer, O. M., *J. Exp. Med.*, 1940, **71**, 495.

<sup>4</sup> Munoz, J. M., Braun-Menendez, E., Fasciolo, J. C., and Leloir, L. F., *Am. J. M. Sc.*, 1940, **200**, 608.

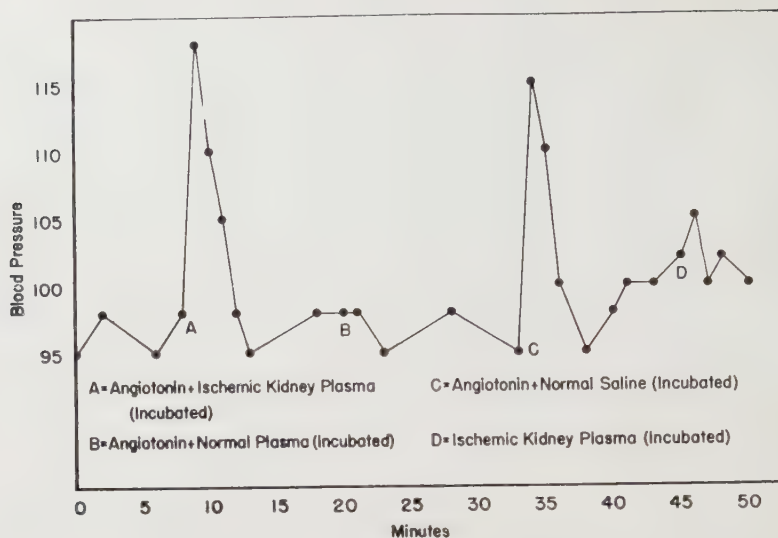


FIG. 1.

*Experiment II-A*—The pressor effect of 5 cc of ischemic kidney blood plasma, 5 cc of normal blood plasma incubated with 0.2 cc of angiotonin solution, and 5 cc of incubated kidney blood plasma.

Although there is strong evidence<sup>1</sup> that purified angiotonin is not exactly similar to the pressor substance found in blood leaving the partially ischemic kidney, it is possible that, because of the extensive chemical procedures necessary for the isolation of angiotonin, certain chemical changes might have occurred in the original substance. If, however, angiotonin does represent the essential chemical derivative of the original effector substance leaving the kidney when its hemodynamics have been seriously altered, then the inability of the venous blood plasma leaving this same kidney to neutralize angiotonin indicates that there may be two mechanisms involved in this type of renal derangement. The evidence presented here suggests strongly that one of these mechanisms is a decreased ability of the blood leaving such an abnormal kidney to neutralize angiotonin.

*Conclusion.* (1) The ability of normal systemic blood plasma to neutralize angiotonin is markedly decreased after circulation through the partially ischemic kidney.

The angiotonin used in these experiments was graciously supplied to us by Dr. Irvine H. Page.

## 13135

## Dehydration and Ketosis.

EATON M. MACKAY, ARNE N. WICK AND FRANK E. VISSCHER.

*From The Scripps Metabolic Clinic, La Jolla, California.*

The antiketogenic influence of fed protein is well known. This action is presumably dependent upon the excess of antiketogenic metabolites (glucogenic amino acids) over ketogenic metabolites (fatty acid residues from amino acids) which protein may furnish during its catabolism. Mineral acids are antiketogenic when fed<sup>1</sup> and the most reasonable explanation for this in the light of our present knowledge is that more carbohydrate becomes available for oxidation due to the increased rate of protein catabolism induced by the acidosis. The experiments reported here were carried out to determine whether or not the condition of dehydration influences ketosis. Water hunger of such degree that dehydration results has an accelerating influence upon protein catabolism.<sup>2-9</sup>

In Experiment 1 male rats were fasted from the stock diet (Tioga dog pellets), the control groups being allowed water while the dehydration groups received no fluid of any kind. There were 3 rats in each group with an average body weight of 254 g. Twenty-four-hour urine collections were made on each group for the determination of total nitrogen. At the end of every 24-hour period a group of 3 rats from each series was sacrificed for blood ketone<sup>10</sup> and liver glycogen determinations.<sup>11</sup>

Female rats averaging 190 g in weight were used in Experiment 2 in groups of 3. They had been receiving a synthetic diet<sup>12</sup> for some time and were fasted with water allowed *ad lib.* for 48 hours

<sup>1</sup> MacKay, E. M., Wick, A. N., Carne, H. O., and Barnum, C. P., *J. Biol. Chem.*, 1941, **138**, 63.

<sup>2</sup> Nothwang, F., *Arch. f. Hyg.*, 1892, **14**, 272.

<sup>3</sup> Landauer, A., *Maly's Jahresb. f. Thierchemie*, 1894, **24**, 532.

<sup>4</sup> Straub, W., *Z. f. Biol.*, 1899, **37**, 527.

<sup>5</sup> Straub, W., *Ibid.*, 1899, **38**, 538.

<sup>6</sup> Spiegler, A., *Z. f. Biol.*, 1901, **41**, 239.

<sup>7</sup> Dennig, A., *Z. f. diatet. u. Physik. Therap.*, 1898, **1**, 281.

<sup>8</sup> Dennig, A., *Ibid.*, 1899, **2**, 292.

<sup>9</sup> MacKay, L. L., and MacKay, E. M., *Am. J. Physiol.*, 1924, **70**, 394.

<sup>10</sup> Barnes, R. H., and Wick, A. N., *J. Biol. Chem.*, 1940, **133**, 59.

<sup>11</sup> Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

<sup>12</sup> MacKay, E. M., and Callaway, J. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1937, **36**, 406.

TABLE I.

Hr Fasting	Avg of control groups				Avg of dehydration groups			
	*Body wt, g	Urine N, mg/rat/day	†Blood ketone bodies, mg %	Liver glycogen, %	*Body wt, g	Urine N, mg/rat/day	†Blood ketone bodies, mg %	Liver glycogen, %
Experiment 1—Male Rats.								
24	228	144	5.6	.04	224	158	5.5	.04
48	225	153	9.4	.18	220	196	8.2	.25
72	215	111	8.3	.38	204	136	4.1	.67
96	210	100	6.2	.80	185	144	3.1	1.49
Experiment 2—Female Rats.								
24†	185	73	31.3	.07	178	82	21.6	.12
36	185		32.9	.29	170		20.3	.37
48	180	63	36.8	.09	164	77	21.1	.32
60	177		27.9	.27	162		10.8	.88
72	167	68	32.5	.14	157	140	16.5	.43

\*When sacrificed.

†As acetone.

‡These fasting periods followed a preliminary fast of 48 hours.

prior to the fasting periods recorded in Table I. Fasting of both groups was continued during the period observations were made. At the beginning of this both groups received 2 cc each of 5% NaCl solution by stomach tube. The dehydration rats were given no other drinking fluid while the controls were allowed water *ad lib.* as usual.

The data summarized in Table I show the usual increase in urine nitrogen excretion with dehydration, a result of the increased protein catabolism. Accompanying this are higher liver glycogen levels, evidence of more available carbohydrate from protein break-down in the dehydrated animals than in the controls. The dehydrated rats have a uniformly lower degree of ketosis as measured by the blood ketone levels than the normally fasting controls. This reduced ketosis is particularly evident where the differences in liver glycogen level from the controls are most definite. It seems reasonable to suppose that the antiketogenic effect of the dehydration is due to the extra carbohydrate provided by the increased protein break-down but there remains the possibility that a general depression in the metabolism of the dehydrated animals may contribute to the reduction in the ketosis level.

*Summary.* Dehydration is antiketogenic when ketosis is measured by the level of ketone bodies in the blood of fasting albino rats. Protein catabolism is increased during dehydration and it is probable that the larger amount of carbohydrate provided by the protein break-down accounts for the antiketogenesis.



## 13136 P

**Total Cardiac Vibrations in Aged Hearts and in Coronary Disease.**

JOHN R. SMITH AND WILLIAM B. KOUNTZ.

*From the Department of Internal Medicine, Washington University School of Medicine, and the Barnes Hospital, St. Louis.*

By means of the vibrocardiograph<sup>1</sup> it is possible to record all the cardiac vibrations (audible and inaudible) that are transmitted through the chest wall. A study of the vibrocardiograms of 100 normal young adults,<sup>2</sup> taken from the usual auscultation areas, established the presence of dominant vibration groups at the onset of systole and at the second sound. These curves were dissimilar to the usual stethogram, as the vibration groups were modified by low-frequency (inaudible) components with which the sound elements are mixed. In 5% of the tracings there appeared low-frequency waves (approximately 1-5 dv. per sec.), the largest mound occurring in mid-systole and two or more in diastole. These waves were of very low amplitude.

The vibrocardiographic curves of 102 individuals over 55 years of age, with and without evidence of coronary disease, were examined. The cases fell into three groups: those with clinical and electrocardiographic evidence of coronary disease (34 cases); those with objective evidence but with normal electrocardiograms (33 cases); and those with no evidence of heart disease. Cases having definite evidence of coronary disease, despite the presence or absence of electrocardiographic change, all showed a prolongation of the total vibration complex at the onset of systole. Normally this complex averages 0.22 second in duration; in these cases the average length was 0.33 second. In some instances of severe myocardial damage, deflections of low frequency with occasional steep slopes occupied nearly all of systole, and on auscultation the sounds were "impure" and muffled. In most of these tracings large, low-frequency waves appeared in systole and diastole similar to those seen in some normals, except that they were definitely augmented in amplitude, occasionally becoming as tall as the most dominant sound deflections; they were in phase with each other and with the onset of ventricular systole. In 15 of the cases without evidence of heart disease, prolongation of the first vibration complex and the presence of the large,

<sup>1</sup> Kountz, W. B., Gilson, A. S., and Smith, J. R., *Am. Heart J.*, 1940, **20**, 667.

<sup>2</sup> Smith, J. R., Edwards, J. C., and Kountz, W. B., *Am. Heart J.*, 1941, **21**, 228.

slow systolic-diastolic waves were noted. None of these cases had received cardiac therapy of any kind.

The low-frequency (inaudible) vibrations recorded by this method may be due to actual motions of the myocardium. Absolute proof for this is yet lacking. However, the tentative suggestion may be made that as the myocardium is compromised by a diminished coronary flow, and tends to become flaccid, slow vibrations may be provoked in the muscle mass by the violent onset of contraction. Whatever the cause, these slow waves as recorded are constant enough in coronary disease to warrant the suggestion that they may indicate flaccidity and weakness of the heart muscle, and may appear before definite signs of myocardial disease develop.

### 13137 P

#### Glycogen Content of the Human Liver.\*

DUGALD S. MACINTYRE,<sup>†</sup> SVEND PEDERSEN AND WALTER G. MADDOCK. (Introduced by H. B. Lewis.)

*From the Department of Surgery, University of Michigan.*

In recent years much attention has been given to the value of the preoperative diet and its relation to the level of liver glycogen in surgical patients. Unfortunately, little is known about the effect of diet, anesthesia and liver pathology upon the glycogen levels of the human liver. Most of our knowledge has been obtained from analysis of human livers obtained at autopsy or from livers of experimental animals. In order to obtain direct information on liver glycogen levels in surgical patients, it was decided to determine the glycogen content of liver specimens obtained by biopsy, and this program was carried out on a series of 33 patients undergoing laparotomy during the winter of 1939-40.

A small section of the biopsy specimen taken during the operation was reserved for microscopic examination and the remainder, approximately 1 g, was immediately frozen in carbon dioxide snow and ether. While still frozen the specimen was ground very fine and transferred into a weighed centrifuge tube containing potas-

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\* This study was aided by a grant from the Horace H. Rackham School of Graduate Studies.

<sup>†</sup> Captain Dugald S. MacIntyre, United States Army Medical Corps, is stationed at the Beaumont Hospital, El Paso, Texas.

sium hydroxide, then quickly weighed, and placed in a beaker of boiling water. After solution was complete, the glycogen was precipitated and hydrolyzed according to the Good, Kramer, and Somogyi modification<sup>1</sup> of Pflüger's method for liver glycogen. The total reducing carbohydrate was determined on aliquots in duplicate according to Shaffer and Somogyi.<sup>2</sup>

In Table I the glycogen values from 26 cases have been grouped according to the extent of liver damage, as shown by microscopic sections reported by the Department of Pathology, the anesthetic used, and whether or not supplementary glucose was fed. The condition of the liver was reported as normal or as showing advanced damage. In 7 additional cases, most of whom showed moderate damage, no grouping could be made because of a multiplicity of varying conditions. The values in these cases ranged between 1.22-4.65%. The average for Group A is 3.15% but if the highest value of 6.31% in this group is omitted, the average of Group A becomes 2.80%. All the individual values in both Groups B and C are above this figure, and the averages of Groups A and D are almost the same.

*Conclusions.* The glycogen content of biopsy samples of normal livers was highest in patients that were fed glucose prior to operation. When extensive liver damage was present, liver glycogen was low despite glucose feeding.

TABLE I.  
Distribution of Values for Hepatic Glycogen.

	A	B	C	D
No. of cases	10	7	5	4
Range %	1.10-6.31 (—3.85)*	3.19-7.56	3.91-5.75	2.70-3.14
Avg %	3.15 (2.80)*	5.03	4.73	2.86

A—Normal livers, spinal anesthesia, no glucose feedings.

B—Normal livers, spinal anesthesia and oral administration of 200 g of glucose during the 12 hours immediately before the operation.

C—Normal livers, nitrous oxide and ether vapor anesthesia and 200 g of glucose similarly fed.

D—Advanced hepatic damage, spinal anesthesia in 3 and nitrous oxide and ether vapor anesthesia in 1, and 200 g of glucose fed.

\*See text.

<sup>1</sup> Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

<sup>2</sup> Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 695.

**Functional Activity of Smooth Muscle Tumors of the Uterus.\***

CHARLES S. BRYAN AND SHIELDS WARREN.

*From the Laboratory of Pathology, New England Deaconess Hospital.*

The question of the functional activity of the cells composing various types of tumor tissue has always excited much interest. It is a remarkable fact that in some tumors cells which have all the morphological characteristics of normal cells of the parent tissue appear to have lost the characteristic functional ability of their normal relatives. In other cases the tumor cells show normal or enhanced activity. As examples may be cited the functioning and nonfunctioning adenomas of pancreas and thyroid. The ability of small metastatic nodules of chorionepithelioma to produce hormonal effects may be much greater than would be expected from their mass.

Leiomyomas of the uterus contain cells which do not differ morphologically from normal smooth-muscle cells. The following experiments were performed to determine whether or not these cells possess the ability to contract, and whether or not there are qualitative differences between their behavior and that of normal uterine smooth-muscle cells.

*Method.* Uteri containing leiomyomas were obtained in the operating room as soon as they were excised and were wrapped in gauze moistened with saline. Within a few minutes strips of muscle about 2.5 cm in length and 0.5 cm in diameter were excised from the tumors as well as control strips from the uterine wall and immediately immersed in warm Ringer's solution through which a constant stream of oxygen was bubbled. The vessel containing them was then immersed in a constant temperature water bath kept at 38°C and the strips attached by threads to pointers recording on a kymograph. In each experiment one strip of uterine muscle and one strip of leiomyoma were immersed in the same bath and attached to separate needles recording at different levels on the same graph. The lower ends of the muscle strips were anchored with pins to a wooden septum which could be changed easily from one container to another, thus allowing quick changes of medium with interval washing. The stimulating solutions were added directly to the Ringer's solution. The agents in the amounts added to the 250 cc of

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\* Read before the meeting of the American Society for Experimental Pathology, April 18, 1941.

Ringer's solution were: (1) Pituitrin,  $\frac{1}{4}$  to  $\frac{1}{2}$  cc; (2) histamine, 0.5 cc of a 1/1000 solution. These amounts are much larger than the minimum needed to cause contraction and were only roughly measured since the experiments were not of a quantitative nature.

At the conclusion of each experiment the muscle strips were fixed in alcohol-formalin and sectioned and stained after celloidin imbedding. In all cases the strips from leiomyoma showed the typical structure of smooth-muscle cells and connective tissue.

*Results.* The smooth muscle of the so-called fibroid tumor is capable of contracting in response to some of the same stimuli which cause contraction of smooth muscle of the uterine wall. To our knowledge this point has not been previously investigated. A total of 16 specimens were tested. In 4 cases there was complete absence of response in both uterine and leiomyoma strips. Usually examination of the sections of muscle which had failed to contract showed marked edema, fibrosis, degenerative changes, or predominantly cross-sectioned rather than longitudinally sectioned muscle cells with resultant mechanical disadvantage. In one case the uterine strip alone responded and in another only the tumor strip contracted. In the remaining 10 cases some degree of response was obtained from both types of tissue.

Table I shows that uterine muscle responded more constantly to pituitrin, while muscle from leiomyomas seemed much more sensitive to histamine. Uterine muscle strips often seemed refractory to histamine, sometimes contracting later to pituitrin. Although our results were not constant enough to establish this point definitely, it did appear that the reaction to histamine might represent a difference in behavior of the two types of muscle.

In one case the addition of 5 drops of extract of ergot to the bath failed to evoke contraction in a leiomyoma strip which responded immediately afterward to histamine. In another case ergot elicited no response in either uterine or leiomyoma strip, though both responded later to pituitrin.

Due to the fact that the relative number of muscle cells is smaller in the tumor than in uterine muscle, because of the large amount of connective tissue in the tumor, the extent of contraction of strips of tumor is usually less. This difference is magnified by the difficulty in obtaining strips of parallel fibres of any length from the tumor. However, in a few cases we succeeded in obtaining vigorous contractions.

In general it appeared that tissues from younger women responded to stimuli quicker and more vigorously than those from older



TABLE I.

	Uterine muscle		Leiomyoma	
	Responded	Failed to respond	Responded	Failed to respond
Pituitrin	8	4	7	5
Histamine	3	5	6	2
Ergot		1		2

The responses of the two types of tissue from each specimen are classified as positive or negative. The response was considered positive if any sample from the specimen showed activity even though other samples failed to contract. Since some specimens were tested with more than one stimulating agent, the number of responses does not correspond to the number of uteri examined.

women. This tendency was not by any means absolute, though, since little or no contraction was elicited from some tissues from women of less than 35, while often specimens from women older than 45 responded well to stimulation. No correlation could be made with the menstrual phase as determined by clinical history.

We were surprised to find that often the tissues, both uterus and tumor, retained the ability to contract for  $1\frac{1}{2}$  to 2 hours if the specimen was merely kept wrapped in moist gauze. In no case did an excised strip give consistent results for more than an hour. Usually after  $\frac{1}{2}$  to  $\frac{3}{4}$  hour in the bath the uterine strips began to show irregular spontaneous contractions with gradually increasing tone and loss of sensitivity to the usual stimulating agents. The strips of leiomyoma usually did not show this increase in tone in the dying phase.

*Summary and Conclusions.* Tissues from 16 leiomyomas were tested for contractility using normal uterine muscle for control. The stimulating substances used were pituitrin, histamine, and ergot.

In 11 cases the tumor tissue showed definite response. Uterine muscle usually showed increasing tone in the dying phase while the tumor tissue became unresponsive without change in tone.

# Hemorrhagic Skin Reactions in Rabbits with Cutaneous Sensitization to Streptococci and Pneumococci (Shwartzman Phenomenon).\*

D. MURRAY ANGEVINE AND PAUL F. DEGARA. (Introduced by E. L. Opie.)

*From the Department of Pathology, Cornell University Medical College, New York City.*

When intravenous injections of living or heat-killed bacteria were given to skin-sensitive rabbits within 48 hours after an intracutaneous injection of either a homologous broth-filtrate or a heat-killed suspension of microorganisms, hemorrhagic reactions frequently occurred within 24 hours at the site of the skin-test. These lesions varied considerably in intensity, were usually purple or violet and closely resembled the hemorrhagic lesions described by Shwartzman.<sup>1</sup>

Such local hemorrhagic reactions in rabbits have been more often induced by bacterial filtrates than by suspensions. Streptococci usually contain little preparatory factor.<sup>1</sup> Antopol<sup>2</sup> produced hemorrhagic reactions with extracts of various microorganisms, including Type 3 pneumococcus, but Witebsky and Neter<sup>3</sup> reported that intravenous injections of living or dead pneumococci were incapable of eliciting hemorrhage at the site of a previous intracutaneous injection of pneumococcal culture.

Sensitization may influence the development of this reaction.<sup>4, 5</sup> Freund pointed out that hemorrhage develops in the skin and peritoneum of tuberculous but not of non-tuberculous guinea pigs. Fabiani elicited a Shwartzman phenomenon in rabbits that became Arthus positive after the third subcutaneous injection of horse serum but not in rabbits that required 5 injections before they became sensitive to horse serum. Zander<sup>6</sup> demonstrated that capillary fragility is greater in areas of allergic inflammation in the skin of rabbits sen-

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\* Aided by grants from the John and Mary R. Markle Foundation, the Ophthalmological Foundation and the Dazian Foundation for Medical Research.

<sup>1</sup> Shwartzman, G., *Phenomenon of Local Tissue Reactivity*, Paul Hoeber, Inc., New York, 1937.

<sup>2</sup> Antopol, W., *J. Inf. Dis.*, 1937, **61**, 330.

<sup>3</sup> Witebsky, E., and Neter, E., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 187.

<sup>4</sup> Freund, J., *J. Exp. Med.*, 1934, **60**, 669.

<sup>5</sup> Fabiani, G., *Compt. Rend. Soc. Biol.*, 1938, **127**, 536.

<sup>6</sup> Zander, E., *J. Exp. Med.*, 1937, **66**, 637.

sitized to streptococci than in control rabbits. Because of the possible relationship of these hemorrhages to hypersensitivity, further investigations seemed desirable.

Sensitization was induced by a variable number of intracutaneous injections of 0.1 cc of heat-killed indifferent streptococci,<sup>7</sup> or Type 1 pneumococci, or formalin-killed hemolytic streptococci (Strain AB 13)<sup>8</sup> given over periods of from 6 to 140 days. Intracutaneous injections were given daily for 6 days and repeated after an interval of 1 to 2 weeks.

The degree of skin-sensitivity was tested by an intracutaneous injection of 0.1 cc of homologous filtrate or vaccine. The average diameter and height of the reactions at 48 hours are recorded in millimeters.

*Provocative Injection.* In the experiments with indifferent streptococci, heat-killed vaccine was injected intravenously 48 hours after the intracutaneous test-injection. In the experiments with hemolytic streptococci and pneumococci, an 18-hour broth-culture was injected intravenously 24 hours after the intracutaneous test-injection. The degree of hemorrhage 24 hours after the provocative injection was recorded as  $\pm$  to ++++.

Agglutinin and precipitin tests were performed close to the time of the intracutaneous test-injection. There was no correlation between circulating antibodies and the degree of hemorrhage in lesions 24 hours after the provocative injection.

*Experiment 1.* Of the rabbits sensitized with repeated intracutaneous injections of heat-killed indifferent streptococci, only No. 1 (Table I) showed a hemorrhagic lesion after the first provocative injection. A second intravenous injection one week later induced a considerably greater hemorrhagic lesion at the site of the last intracutaneous test-injection. Rabbits No. 2 and No. 6 that did not react to the first provocative injection also gave strongly positive skin-reactions after the second intravenous injection. There was no definite correlation between skin-sensitivity and degree of hemorrhage in these animals. However, the most intense reactions were observed in the 2 animals that had the highest degree of cutaneous sensitivity at the time of the second test.

*Experiment 2.* In 6 of 9 rabbits sensitized with hemolytic streptococci, intense hemorrhagic lesions were observed after the intravenous injection of 2 cc of homologous broth-culture (Table II). There was no correlation between skin-sensitivity and degree of

<sup>7</sup> McEwen, C., and Swift, H. F., *J. Exp. Med.*, 1935, **62**, 573.

<sup>8</sup> Nichols, E. E., and Stainsby, W. J., *J. Clin. Invest.*, 1931, **10**, 325.

TABLE I.

Degree of Hemorrhage in Rabbits Sensitized by Repeated Intracutaneous Injections of Heat-Killed Indifferent Streptococci.

Rabbit No.	No. of injections	Degree of skin-sensitivity to filtrate 143d day	Degree of hemorrhage in lesion 24 hr after i.v. injection of 1 cc of heat-killed streptococci 145th day	Degree of skin-sensitivity to filtrate 150th day	Degree of hemorrhage in lesion 24 hr after i.v. injection of 1 cc of heat-killed streptococci on 152d day
1	50 during	39.2	++	35.2	++++
2	140 days	30.1	None	37.2	+++
3		30.1	"	30.2	None
4		40.2	"	33.1	"
5		23.1	"	29.1	"
6		22.1	"	25.0	++

hemorrhage. Ten normal rabbits were given an intracutaneous injection of streptococcal filtrate; then after 48 hours they were injected intravenously with 2 cc of homologous culture; no hemorrhage was observed at the site of intracutaneous injection.

*Experiment 3.* The experiments with Type 1 pneumococci are summarized in Table III. After the provocative injection of 1 cc of broth culture, intense hemorrhage was observed in the skin of rabbit No. 24. This was the most sensitive animal of the group. Hemorrhages of lesser degree were observed in rabbits Nos. 19, 22, 23, and 25. In this group the intensity of the hemorrhagic reaction was greater in rabbits which had been sensitized by a larger number of intracutaneous injections, and had reached a higher degree of cutaneous sensitivity to the vaccine.

*Summary.* When an intracutaneous injection of bacterial filtrates or heat-killed organisms into sensitized rabbits was followed

TABLE II.

Degree of Hemorrhage in Rabbits Sensitized by Repeated Intracutaneous injections of Formalin-killed Hemolytic Streptococci.

Rabbit No.	No. of injections	Degree of skin-sensitivity to filtrate	Degree of hemorrhage in lesion 24 hr after i.v. injection of 2 cc of streptococcal broth culture
7	24 during 42 days	70.3	None
8		67.3	++++
9		60.3	++++
10		46.2	+++
11		40.2	+++
12		40.2	None
13		32.2	++++
14		40.1	++++
15		40.0	None

TABLE III.  
Degree of Hemorrhage in Rabbits Sensitized by Repeated Intracutaneous injections  
of Heat-killed Pneumococci Type I.

Rabbit No.	No. of injections	Duration of sensitization (days)	Degree of skin-sensitivity to vaccine	Degree of hemorrhage in lesion 24 hr after i.v. injection of 1 cc of pneumococcal broth culture
16	6	6	7-1	None
17	6	6	4-1	"
18	12	20	23-2	"
19	12	20	16-2	+
20	12	20	4-0	None
21	18	34	29-3	"
22	18	34	21-3	±
23	18	34	29-2	±
24	24	48	31-3	+++
25	24	48	28-3	+
26	24	48	18-2	None

by intravenous injections of sufficient amounts of killed or living organisms, a hemorrhagic reaction frequently developed at the site of the intracutaneous injection.

The most intense hemorrhagic reactions were observed in animals with the highest degree of cutaneous sensitization. We did not observe hemorrhagic phenomena in non-sensitized animals treated in a similar manner.

### 13140

#### Blood Pantothenic Acid Values in Multiple Sclerosis.

LOUIS SIEGEL, TRACY J. PUTNAM AND JOHN G. LYNN. (Introduced by W. W. Palmer.)

*From the Food Research Laboratories, Long Island City, New York, and the Department of Neurology, College of Physicians and Surgeons, Columbia University, and the Neurological Institute, New York City.*

Since the discovery and synthesis of pantothenic acid (Williams and Major<sup>1</sup>), many attempts have been made to determine its clinical value. Stanbery, Snell and Spies<sup>2</sup> have shown that in deficiencies of the factors of the vitamin B complex, there is always a decrease in the blood pantothenic acid values. Their normal subjects gave values from 19.0 to 32.0  $\mu\text{g } \%$ , with an average of 22.5. The daily varia-

<sup>1</sup> Williams, R. J., and Major, R. T., *Science*, 1940, **91**, 246.

<sup>2</sup> Stanbery, S. R., Snell, E. E., and Spies, T. D., *J. Biol. Chem.*, 1940, **135**, 353.



tion was within 2.0  $\mu\text{g } \%$ . In cases of deficiency, such as pellagra, beri beri and aflavinosis, the blood pantothenic acid values were found to be from 50 to 77% of the average normal figure.

Phillips and Engel<sup>3</sup> demonstrated that in pantothenic acid-deficient chicks there were lesions of the spinal cord characterized by degeneration of the myelinated fibers. In longitudinal sections degenerative changes were frequently observed in the axis cylinders of the involved nerve.

In multiple sclerosis pathological changes are found which despite a greater relative degeneration of myelin sheaths as compared to axis cylinders have certain superficial resemblances to the lesions described by Phillips and Engel. This suggested to the first author that a deficiency of pantothenic acid might constitute at least a contributing factor in the development of this disease. Accordingly, analyses for this vitamin were conducted on blood of normal and multiple sclerotic individuals to ascertain whether any differences existed.

The microbiological method of Pennington, Snell and Williams<sup>4</sup> as subsequently modified<sup>2</sup> was used. The normal subjects were hospital and laboratory personnel. The clinical material was obtained from patients admitted to the Neurological Institute, and who were diagnosed as advanced cases of multiple sclerosis. In these cases the disease was from 2 to 25 years' duration and in various stages of remissions and relapses. One case of amyotrophic lateral sclerosis was also studied. All subjects subsisted on normal adequate diets, precautions being taken that foods rich in pantothenic acid such as liver and molasses were excluded.

The blood analyses were run in duplicate. A survey of the results indicated that in our hands the method shows a technical reliability or consistency reflected in values reproducible to within 3.5% of the average figure with a maximal deviation of 9.5%.

In Table I the results of the present study are summarized.

Our normal subjects had blood pantothenic acid values varying from 19.7 to 33.5  $\mu\text{g } \%$ ; this is in close agreement with the range of values reported by Stanbery, Snell and Spies. The blood values of the multiple sclerotic subjects were also in the normal range. This indicates that pantothenic acid deficiency can not be regarded as having etiologic significance in this disease.

In one case of multiple sclerosis, subject B.S., the dietary history

<sup>3</sup> Phillips, P. H., and Engel, R. W., *J. Nutrition*, 1939, **18**, 227.

<sup>4</sup> Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1940, **135**, 213.

TABLE I.  
Blood Pantothenic Acid Values in Normal and Multiple Sclerotic Individuals.

Subject	Clinical status	Blood pantothenic acid level $\mu\text{g}\%$	
H.H.	Normal	19.7	
J.G.	"	29.4	Mean = 28.1
M.W.	"	29.3	Avg deviation
D.M.	"	33.3	= 2.2
H.D.	"	23.5	
H.S.	"	33.6	
I.B.	Multiple Sclerotic	29.6	
B.S.	" "	19.7	Mean = 28.8
E.F.	" "	28.0	Avg deviation
J.P.	" "	33.6	= 3.3
K.K.	" "	32.8	
H.S.	" "	29.0	
S.O.	Amyotrophic lateral sclerosis	32.0	

indicated a low intake of pantothenic acid. Oral administration of increased quantities of pantothenic acid in the form of food rich in this vitamin led to a prompt increase in the blood value approximating the maximal in the normal group. This observation agrees with that reported by Spies and associates<sup>5</sup> and supports their conclusion that pantothenic acid deficiency is associated with a decrease in the blood value.

*Summary.* Blood pantothenic acid values from six normal subjects varied in the present study over the usually obtained normal range of from 19.7 to 33.6  $\mu\text{g}\%$ . These values are ordinarily decreased in cases of pantothenic acid deficiency. Six patients with advanced multiple sclerosis failed to show any such decrease in the concentration of pantothenic acid in the blood indicating that this vitamin is probably not an etiologic factor in this disease.

<sup>5</sup>Spies, T. D., Hightower, D. P., and Hubbard, L. H., *J. A. M. A.*, 1940, **115**, 292.

## 13141

## Importance of Choline in Synthetic Rations for Dogs.\*

A. E. SCHAEFER, J. M. MCKIBBIN AND C. A. ELVEHJEM.

*From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.*

During studies on the newer members of the vitamin B complex with dogs<sup>1, 2</sup> we observed that a highly purified ration supplemented with synthetic thiamin, riboflavin, nicotinic acid, pyridoxine, and pantothenic acid does not permit survival. Evidence was presented for the existence of two other factors present in liver extract essential for the dog. Since choline was found to be essential for the prevention of fatty livers in depancreatized dogs,<sup>3</sup> we suspected very early that choline may have been one of these factors. The administration of choline, however, produced no effect in the deficient animals and it was concluded that these factors were distinct from choline. But in the light of more recent work with both rats and chicks we must conclude that the dose used was far below that found necessary for these other animals. We have, therefore, re-studied the significance of choline in the nutrition of the dog using higher levels of choline.

Two litters of mongrel puppies were used in these experiments. The ration, as previously described, contained sucrose 66%, acid washed casein 19%, cottonseed oil 8%, cod liver oil 3%, and salt mixture 4%. This was supplemented with thiamin and riboflavin at a level of 100  $\gamma$  per kg of body weight per day, nicotinic acid at a level of 2 mg, pyridoxine at 60  $\gamma$ , and calcium pantothenate at 500  $\gamma$  per kilo per day. These vitamins were administered orally in water solution twice weekly. Dogs 1, 2, 3, 4, and 5 were placed on this ration at the age of 8 weeks. Dogs 6, 7, and 8 of the other litter

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<sup>1</sup> McKibbin, J. M., Madden, R. J., Black, S., and Elvehjem, C. A., *Am. J. Physiol.*, 1939, **128**, 102.

<sup>2</sup> McKibbin, J. M., Black, S., and Elvehjem, C. A., *Am. J. Physiol.*, 1940, **130**, 365.

<sup>3</sup> Best, C. H., and Ridout, J. H., *Ann. Rev. Biochem.*, 1939, p. 349.

were placed on the ration at 6 weeks of age. The growth curves of these dogs are shown on the graph. It can be seen that dogs 1, 2, 3, 4, 5, and 8 receiving the choline-low ration showed a quick growth plateau followed by anorexia and loss of weight. No other symptoms were observed. The administration of choline at a dosage of 100 mg per kg of body weight per day produced an immediate growth response in these dogs. Dogs 6 and 7 received 50 mg of choline per kg per day from the beginning of the experiment and serve as positive controls. These animals gained 1.85 and 2.1 kg, respectively, while the litter mate dog No. 8 had gained only 0.9 kg during the 25-day period. The addition of choline to the diet of dog No. 8 resulted in a weight increase of 1.75 kg in 12 days. The condition of the animals at the time of treatment was more critical than would appear from the growth curves. It has been our experience with puppies on this ration that prolongation of the anorexia is usually fatal. It is possible that difficulties of this kind encountered in our earlier studies were at least in part due to choline deficiency. We are now studying the choline content of the various liver fractions used to determine the relation of choline to the activity of these fractions. A high incidence of yellow livers observed in these dogs suggests a parallel to those found in choline-deficient depancreatized dogs.

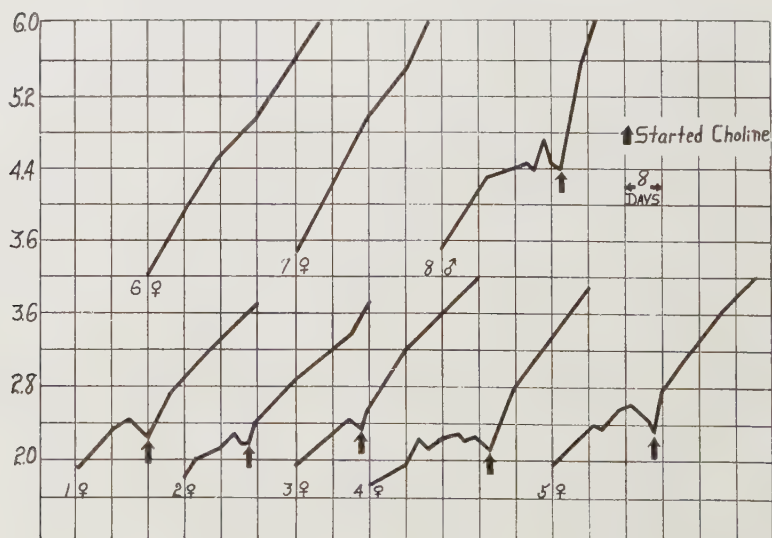


FIG. 1.

Growth curves of young dogs on the basal synthetic ration with and without choline.

The speed of onset of the deficiency is comparable to that of the other B vitamins. We have found in extensive studies on this ration involving some 128 dogs over a 3-year period that acute fatal deficiencies of thiamin, riboflavin, nicotinic acid, pyridoxine, and probably pantothenic acid may be expected within 2 to 6 weeks. This is in distinct opposition to the claim of Morgan<sup>4</sup> that "Even in very young animals several months are required for the development of overt symptoms of deficiency in any of the B vitamins, with the possible exception of B<sub>1</sub>."

The requirement of choline may be supposed to be largely dependent on the amounts of methionine and cystine in the diet.<sup>5, 6, 7</sup> Increasing the level of casein, therefore, lowers the choline requirement of rats. Griffith<sup>6</sup> found that the hemorrhagic degeneration of choline deficiency in rats was prevented by a low fat diet containing 47% casein even in the presence of 0.5% cystine. Most other laboratories have used higher levels of casein than we have used and this might entirely nullify the requirement for choline. Thus Fouts, *et al.*,<sup>8</sup> used 41.4%, Borson and Mettier,<sup>9</sup> 37% casein in their vitamin B<sub>6</sub> deficiency studies with dogs. In addition, the natural vitamin B complex sources such as liver extract and rice bran preparations contain considerable quantities of choline. The choline would have to be removed from these preparations in order to demonstrate this deficiency.

The growth rate of puppies maintained on this ration supplemented with thiamin, riboflavin, nicotinic acid, pyridoxine, and pantothenic acid is very suboptimal and erratic even with added choline. The ration is, therefore, still not entirely satisfactory for studying the uncomplicated deficiency of any of the 6 synthetic vitamins without the addition of a crude vitamin B complex preparation, although we have used a ration of this kind for nicotinic acid assay of foods over limited periods of time. Studies on other substances in liver extract necessary for the normal growth of the dog are now in progress.

*Summary.* In addition to thiamin, riboflavin, nicotinic acid, pyridoxine, and pantothenic acid, young puppies also require choline

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<sup>4</sup> Morgan, A., *Science*, 1941, **93**, 261.

<sup>5</sup> du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, 1939, **131**, 57.

<sup>6</sup> Griffith, W. H., *J. Biol. Chem.*, 1940, **132**, 639.

<sup>7</sup> Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, 1940, **132**, 627.

<sup>8</sup> Fouts, P. J., Helmer, O. M., and Lepkovsky, S., *J. Nutr.*, 1940, **19**, 393.

<sup>9</sup> Borson, H. J., and Mettier, S. R., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 429.



and probably other factors of the vitamin B complex for normal growth under the conditions of these studies. Possible factors altering the requirement of choline for dogs on synthetic rations are discussed.

## 13142

### Isolation and Pure Cultivation of the Smaller Mouth Spirochetes by an Improved Method.

THEODOR ROSEBURY AND GENEVIEVE FOLEY. (Introduced by A. R. Dochez.)

*From the Department of Bacteriology, Columbia University, New York.*

The characteristics and properties of the so-called commensal spirochetes of mucous membranes, and their individual or contributing rôles in fuso-spirochetal infections, remain inadequately understood largely because of a lack of effective means for their isolation and maintenance in pure culture. *Treponema vincenti*, the large delicate loosely wound spirochete of the mouth, seems never to have been obtained in pure culture; *T. buccale*, the large, thick, loosely wound form, has been reported in culture rarely,<sup>1, 2, 3</sup> apparently under conditions which could not be duplicated. *T. microdentium*, the small mouth spirochete which resembles *T. pallidum* in morphology and is cultivable with the least difficulty,<sup>4-8</sup> seems for that reason to be the only member of the group which can be accepted with little doubt as a distinct species. Studies of pure cultures of *T. microdentium*, moreover, have yielded interesting information on the pathogenesis of fuso-spirochetal infections.<sup>5, 6, 9, 2</sup> On the other hand, none of the other varieties which have been described and named, such as Noguchi's *T. macrodentium*<sup>4</sup> or the several species named by Seguin and Vincent,<sup>10</sup> is so clearly defined.

<sup>1</sup> Smith, D. T., *Oral Spirochetes and Related Organisms in Fuso-Spirochetal Disease*, Baltimore, Williams and Wilkins Co., 1932, p. 11.

<sup>2</sup> Proske, H. O., and Sayers, R. R., *U. S. Pub. Health Rep.*, 1934, **49**, 839, 1212.

<sup>3</sup> Vincent, R., and Daufresne, M., *Compt. rend. Soc. Biol.*, 1934, **116**, 490.

<sup>4</sup> Noguchi, H., *J. Exp. Med.*, 1912, **15**, 81.

<sup>5</sup> Kritchewski, B., and Seguin, P., *Rev. stomatol.*, 1920, **22**, 613.

<sup>6</sup> Smith, D. T., *Am. Rev. Tuberculosis*, 1927, **16**, 584.

<sup>7</sup> Smith, D. T., *loc. cit.*, 1932, p. 8.

<sup>8</sup> Seguin, P., and Vincent, R., *Ann. de l'Inst. Pasteur*, 1938, **61**, 255.

<sup>9</sup> Smith, D. T., *loc. cit.*, 1932, p. 124.

<sup>10</sup> Seguin, P., and Vincent, R., *Compt. rend. Soc. Biol.*, 1936, **121**, 408.

Most successful attempts to cultivate the smaller commensal spirochetes<sup>4-8, 9, 10, 11</sup> have utilized methods based on those developed by Noguchi.<sup>4, 12</sup> These methods are laborious and intricate, particularly in that the steps necessary to obtain pure cultures from the invariably mixed source material are often discouragingly difficult, as exemplified in a recent report by Kast and Kolmer.<sup>13</sup> Experiences with the older methods in this laboratory<sup>14</sup> have led to the development of a modified technic which has greatly simplified what had been the most difficult phase of this work, namely, the isolation of spirochetes from a primary mixed culture.

The distinguishing feature of the method for primary isolation lies in the use, in place of Noguchi's deep tubes, of small Petri plates containing a thick layer of solid medium, which are inoculated by stabbing into the side of a well previously cut in the center of the plate, and incubated in anaerobic jars. Two sizes of plates have been used, 50 x 14 mm (overall) and 60 x 18 mm, either of which is satisfactory if uniform in depth. About 10 cc of medium is placed in the smaller plates and 20 cc in the larger, so that the depth of medium in the center of the plate is at least 7 mm. Noguchi's solid media<sup>12</sup> may be used, enriched with 1 part of sterile serum or ascitic fluid to 2 parts of agar base and containing fresh sterile tissue, the tissue, however, being minced fine and distributed on the floor of the plate instead of being added in a single piece as in tubes. In place of the usual enriching fluids, serum ultrafiltrate, as used by Simms and Stillman<sup>15,\*</sup> for tissue culture, and by Sanders<sup>16</sup> and Molloy<sup>17</sup> for the cultivation of several viruses, has been found more satisfactory, in that repeated batches have yielded consistently effective media. Occasional batches of ascitic fluid have yielded somewhat wider

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11 Vincent, R., Seguin, P., and Daufresne, M., *ibid.*, 1936, **121**, 406; Seguin, P., and Vincent, R., *ibid.*, 1936, **121**, 488; Vincent, R., and Seguin, P., *ibid.*, 1939, **130**, 12; *Bull. de l'Acad. Med.*, 1939, **121**, 407.

12 Noguchi, H., *J. Exp. Med.*, 1912, **15**, 90, 466; 1912, **16**, 194; 1913, **17**, 89.

13 Kast, C. C., and Kolmer, J. A., *Am. J. Syph.*, 1940, **24**, 671.

14 Rights, F. L., unpublished data.

15 Simms, H. S., and Stillman, N. P., *J. Gen. Physiol.*, 1937, **20**, 649; *Arch. Path.*, 1937, **23**, 316, 322.

\* We are grateful to Dr. Simms for his interest in these experiments and for supplying us with serum ultrafiltrate in the early part of this work, and to Warner Institute for Therapeutic Research, 113 West 18th Street, New York City, for supplying the ultrafiltrate more recently. This institute is now prepared to supply serum ultrafiltrate made according to Dr. Simms' directions.

16 Sanders, M., *J. Exp. Med.*, 1940, **71**, 113; Sanders, M., and Molloy, E., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 327.

17 Molloy, E., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 563.

zones of spirochetal growth than comparable media containing serum ultrafiltrate, but other batches of ascitic fluid have been worthless.

The agar base used in these studies, both for isolation and for maintenance of pure cultures, is the veal heart agar of Proske and Sayers,<sup>2</sup> without modification. This agar base may be prepared in large batches and stored in the refrigerator for several months. Sterile serum ultrafiltrate may likewise be stored in sealed containers for at least two months.

The tissue used in most of these experiments has been guinea pig kidney; of the other tissues tried, guinea pig testicle seemed less effective, and rabbit kidney or testicle were not more effective. A guinea pig is exsanguinated and the kidneys removed aseptically and carefully freed from capsular connective tissue and fat. The two kidneys are then minced fine—so that the largest particles are about 2 mm wide—in 10 cc of serum ultrafiltrate diluted 1 part with 2 parts of Simms' salt solution.<sup>18</sup> Simms and Sanders<sup>18</sup> report that the cells in such finely minced adult tissues remain healthy for long periods in diluted serum ultrafiltrate, whereas larger fragments rapidly become necrotic at the center. About 0.1 cc of the suspension of minced kidney is used for each 10 cc of final medium. The tissue is transferred to the plates first with a sterile wide-tip pipette or glass tube. The agar base, liquefied and cooled to about 50°C, is then added to undiluted serum ultrafiltrate (warmed to about 40°C) in the proportion 2 parts of agar base to 1 part of ultrafiltrate, and the mixture poured into the plates over the minced kidney in such a way as to distribute the tissue particles uniformly. Plates are incubated aerobically at 37°C overnight, and then anaerobically at 37°C for an additional 24 hours or more to assure sterility before use. The plates may subsequently be stored in air at room temperature for at least 2 weeks without impairment of their ability to grow spirochetes.

Just before use a well is cut in the center of each plate with a pipette drawn from thin-walled glass tubing so that the diameter of the tip is about 2 mm. The well should preferably not reach the bottom of the plate, and care must be used to avoid undercutting the agar. A single stab inoculation is made with a straight platinum needle to a depth of about 2 mm obliquely into one side of the well, in such a way that neither the upper nor the lower surfaces of the agar are touched by the inoculum. In general, and particularly when a concentrated inoculum such as undiluted exudate is used, three or more plates should be stabbed serially without recharging the

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<sup>18</sup> Simms, H. S., and Sanders, M., to be published.

needle. Disruption of the medium by gas-forming bacteria is rarely seen in these plates because the well provides a pathway of escape for gas; but such disruption—one of the common difficulties with the tube method—may occur if the inoculum is too heavy or stabbed too deeply.

The inoculated plates are incubated at 37°C inverted in jars made anaerobic with hydrogen, catalyzed by electrically heated platinized asbestos, and containing 5% of carbon dioxide. Spirochetes begin to appear on about the 4th day in primary plates, and are ready for examination and transfer on the 5th to the 7th day.

In a successful primary plate bacterial contaminants are limited chiefly to the immediate vicinity of the stab and to the sides of the well, and do not extend more than a few millimeters over the surface of the plate from the well edge. The spirochetes, on the other hand, grow in the depths of the medium as a clearly visible haze extending well beyond the surface bacterial growth under the sterile agar surface. They can, therefore, be obtained free from bacteria merely by removing a portion of the infiltrated agar through the sterile surface at a distance from the bacterial growth.

By the use of this method of primary isolation, spirochetal growth accessible without contamination has been obtained in plates from 23 of a total of 29 clinic cases, chiefly fuso-spirochetal mouth infections.<sup>†</sup> Of the 6 failures one was a case of fuso-spirochetal empyema in which exudate taken at operation showed no spirochetes under darkfield illumination but nevertheless induced a typical fuso-spirochetal response when inoculated subcutaneously into a guinea pig. The other 5 samples which yielded negative cultures were also either poor in spirochetes originally, or had been allowed to stand before inoculation until motility was lost. It is interesting that primary plates inoculated with exudates from experimental fuso-spirochetal infection in guinea pigs<sup>19</sup> have been successful from all of the 21 animals tested.

These findings indicate the positive nature of the method of primary isolation. On the other hand, whereas mixed bacterial and spirochetal growth can easily be subcultured repeatedly merely by transferring mixed growth from the central well with a straight needle to similarly prepared sterile plates, successful pure subculture of spirochetes is more difficult. Pure cultures have been obtained

<sup>†</sup> Heavy suspensions of gingival scrapings in 1 cc of broth were prepared for this purpose through the courtesy of Dr. D. E. Ziskin and his staff in the School of Dental and Oral Surgery, Columbia University.

<sup>19</sup> Rosebury, T., and Foley, G., *J. Am. Dent. Assn.*, 1939, **26**, 1798.





FIGS. 1, 2, 3.

Primary plates inoculated by stab in central well with fuscospirochetal exudates and incubated anaerobically at 37°C for 5 to 7 days. Scattered particles are fragments of minced guinea pig kidney; lighter sector in upper portion of Fig. 1 is kidney sediment. Mixed bacterial growth is present around the central well, with spirochetal growth extending beyond it in the depths of the medium. The three plates show different types of spirochetal growth.



FIG. 4.

Pure plate culture of *Treponema microdentium* incubated 7 days anaerobically at 37°C. Kidney fragments are clumped chiefly in the upper portion. The vertical white line at the center of the area of spirochete growth is the residue of the inoculum, which apparently increases in density during incubation.

FIG. 5.

Four pure tube cultures of spirochetes, incubated 7 days anaerobically at 37°C. The vertical white line in each is the inoculum, intensified during growth; the infiltrating hazes of spirochetal growth show different characteristics in the 4 tubes.

in plates, using the same medium without wells, by transferring a column of spirochete-infiltrated agar with a sterile capillary pipette. Spirochetes growing nearest to the bacteria in the well seem to be most viable; hence the inoculum should be taken from an area as close to the well as is possible without contamination. The inoculum is introduced under the surface of the fresh plate by blowing through a rubber tube attached to the pipette, with care to avoid introducing air bubbles or otherwise breaking the agar surface. Successful pure subcultures have been obtained only when such transfers were made immediately after removing the primary plates from the anaerobic jar, without taking time, for example, to examine a portion of the inoculum under the darkfield microscope until after the transfers had been made and placed under anaerobiosis. Pure subcultures in plates have not been so consistently successful as subcultures from the primary plates into tubes. For this purpose the same medium is employed, using 5 cc in Wassermann tubes, except that a single piece of guinea pig kidney is used in the bottom of the tube in place of the minced tissue. One guinea pig kidney may be cut into at least 20 pieces for this purpose. Again the transfer is made with a capillary pipette, the inoculum being drawn up with the aid of a rubber tube as an unbroken column about 2 cm or more in length, and carefully expelled without bubbles as the pipette is withdrawn in the vertical axis of the tube beginning a few millimeters above the kidney fragment. The tubes, like the plates, are incubated in anaerobic jars under hydrogen and 5% CO<sub>2</sub>. Pure cultures grow more slowly than primary cultures, requiring 7 to 9 days at 37°C; the spirochetes are then easily removed with capillary pipettes through the upper surface of the medium. This method of pure subculture has been applied only during the past few weeks. In that time 11 strains of small mouth spirochetes, including *T. microdentium* and other as yet unidentified varieties, have been isolated and passed successfully through as many as 7 subcultures. These studies are being continued.

*Summary.* Details are given of a method for primary anaerobic cultivation in small plates of spirochetes from human and experimental fuso-spirochetal exudates, and for their pure subculture in

plates and tubes. Successful primary plates were obtained in 23 instances out of 29 cases of human infection, and from all of 21 experimentally infected guinea pigs. Eleven strains of spirochetes have been isolated and subcultured in a short period with little difficulty.

### 13143 P

#### Comparative Amino Acid Content of Serum Proteins in Normal Humans and in Patients with Rheumatoid Arthritis.

WALTER D. BLOCK AND WILLIAM A. MURRILL.\* (Introduced by H. B. Lewis.)

*From the Rackham Arthritis Research Unit,† the Medical School, University of Michigan, Ann Arbor, Mich.*

The existence of a disturbance in sulfur metabolism in patients with chronic rheumatoid arthritis has been emphasized by Wheel-  
don.<sup>1</sup> Race<sup>2</sup> has extended this concept of disturbed sulfur metabolism to include an alteration in the cystine content of serum proteins. In order to test the validity of this finding the determination of several amino acids, as well as the elemental analyses of total serum protein, were carried out in 11 patients with chronic rheumatoid arthritis and compared with the findings in 6 normal control subjects.

*Procedure.* Fifty cc of venous blood were withdrawn from each subject (150 cc in the cases of control No. 6 and arthritic No. 11). The serum was separated and the total serum protein precipitated with 2 volumes of alcohol. The precipitate was dialyzed until it was sulfate and chloride free. It was then washed several times with 95% alcohol, once with absolute alcohol and several times with ether, and dried in a vacuum desiccator over sulfuric acid for several days. The analytical methods for total nitrogen, total sulfur and the various amino acids were those previously employed by Block and Lewis.<sup>3</sup> The values for serum protein were corrected for ash and moisture

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\* Research Fellow in Internal Medicine, 1940-1941.

† The Rackham Arthritis Research Unit is supported by the Horace H. Rackham School of Graduate Studies, of the University of Michigan.

<sup>1</sup> Wheel-  
don, T., *J. Bone and Joint Surg.*, 1935, **17**, 693.

<sup>2</sup> Race, J., *Reports on Chronic Rheumatic Diseases*, H. K. Lewis & Co., Ltd., London, 1935, **1**, 55.

<sup>3</sup> Block, W. D., and Lewis, H. B., *J. Biol. Chem.*, 1938, **125**, 561.

TABLE I.  
Analyses of Normal Human and Arthritic Total Serum Protein.

Subjects	Age, years	Sex	Nitrogen %	Sulfur %	Cystine %	Tyrosine %	Tryptophane %
Normal Controls:							
1	23	F	15.08	1.46	3.33	4.84	1.06
2	22	F	15.38	1.23	3.29	4.63	1.42
3	35	M	15.32	1.36	3.36	4.49	1.32
4	28	F	15.88	1.29		4.28	1.34
5	35	M	15.45	1.18	3.26	4.50	1.30
6	22	M	15.28	1.51	3.86	4.56	1.47
Avg	27		15.40	1.34	3.42	4.55	1.32
Rheumatoid Arthritics:							
1	35	M	15.08	1.17	3.36	4.86	1.46
2	55	F	15.14	1.41	3.13	4.93	1.42
3	42	M	15.24	1.50	3.67	5.15	1.42
4	29	M	14.12	1.32	3.51	5.27	1.42
5	56	F	15.38	1.24	3.37	4.83	1.46
6	28	F	15.33	1.29	3.42	4.99	1.23
7	30	M	15.38	1.18	3.16	4.82	1.45
8	51	M	15.10	1.61	3.41	4.94	1.41
9	46	F	15.05	1.42	3.51	4.80	1.40
10	53	M	15.11	1.21	3.30	4.83	1.43
11	26	F	15.38	1.39	3.28	5.15	1.40
Avg	41		15.18	1.34	3.37	4.96	1.41

All values except the total nitrogen are corrected for ash and moisture as described in the text.

by the following procedure. Assuming 16% as the average nitrogen content for serum protein corrected for ash and moisture, our various analytical values were multiplied by the ratio of 16 over the determined nitrogen content.

*Results.* The results are summarized in Table I. Both the individual and average values for nitrogen and sulfur as well as for the individual amino acids are essentially the same for the arthritic and control subjects. These results, especially in regard to total sulfur and cystine content are contrary to the suggested findings of Race<sup>2</sup> and indicate no alteration in the composition of the serum proteins of arthritic patients. This is well demonstrated by the average cystine content of 3.42% in the serum protein of normal controls as compared with average value of 3.37% in the arthritic subjects. The values of 5.2% arginine, 2.5% histidine, and 9.6% lysine obtained from the total serum proteins of normal controls likewise were not appreciably different from values of 4.9%, 2.1% and 8.4% for the corresponding basic amino acids of the total serum protein secured from arthritic subjects. Furthermore, the average values for the other amino acids in the total serum protein of arthritics

show no essential deviation from those found in the total serum protein of normal individuals.

*Conclusions.* The total sulfur, total nitrogen and the amino acid content of total serum proteins from normal and arthritic subjects were compared. No essential difference in values between the two groups could be found, invalidating any suggestion that an altered composition of the total serum protein of arthritic patients reflects a disturbed sulfur metabolism.

### 13144 P

#### Inositol in Chick Nutrition.\*

D. M. HEGSTED, G. M. BRIGGS, R. C. MILLS, C. A. ELVEHJEM AND  
E. B. HART.

*From the Department of Biochemistry, College of Agriculture, University of  
Wisconsin, Madison, Wis.*

Eastwood<sup>1</sup> showed inositol to be one of the bios factors required by certain yeasts. Recently Woolley<sup>2, 3</sup> has demonstrated that the mouse requires inositol for normal growth and the prevention of alopecia. Pavcek and Baum<sup>4</sup> have also found inositol to be effective in preventing lack of growth and "spectacled eye" in rats maintained on certain purified diets. These results together with the isolation of phytic acid from chicken blood by Rapoport<sup>5</sup> suggested that the chick might also require inositol.

Growth responses varying from 18 to 52 g in 4 weeks have been obtained upon the addition of inositol<sup>†</sup> to several different simplified chick rations (Table I). It is clear that inositol has a definite growth-promoting action although the response varies with the amount of inositol in the ration and the adequacy of the supplement used to supply unidentified growth factors.

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\* This work was supported in part by grants from the Wisconsin Alumni Research Foundation and the Works Progress Administration.

<sup>1</sup> Eastwood, E. V., *J. Phys. Chem.*, 1928, **32**, 1094.

<sup>2</sup> Woolley, D. W., *Science*, 1940, **92**, 384.

<sup>3</sup> Woolley, D. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1941, **46**, 565.

<sup>4</sup> Pavcek, P. L., and Baum, H. M., *Abst. of Papers, St. Louis Meeting, Am. Chem. Soc.*, April, 1941.

<sup>5</sup> Rapoport, S., *J. Biol. Chem.*, 1940, **135**, 403.

<sup>†</sup> i-inositol, C.P., Pfanstiehl.

TABLE I.  
 Growth Responses Obtained with Inositol.

Group	Ration	No. of chicks	Avg wt at 4 wks g
1	477	22	148
	" + .1% inositol	18	166
2	477-KR + 10% molasses	5	76
	" + " " + .1% inositol	5	121
3	" + " " + SLE eluate	11	167
	" + " " + " " + .1% inositol	11	203
4	477 + SLE eluate	6	156
	" + " " + .1% inositol	6	208
5	477 + alc. sol. yeast eluate	12	162
	" + " " " " + .1% inositol	12	196

Ration 477 is the same as ration 470 described previously<sup>6</sup> with the following additions: 3% of extracted kidney residue, .15% choline, 15 mg of pantothenic acid, 100 mg of nicotinic acid, and 4 mg of pyridoxine per kilo. The thiamine and riboflavin content have been raised to 3 and 4 mg per kilo respectively and additional  $\text{MnSO}_4$  has been added to raise the level of manganese to approximately 60 p.p.m. Ten percent of blackstrap molasses replaced kidney residue in groups 2 and 3 as a source of the antidermatitis factor previously studied.<sup>7</sup> SLE eluate designates a norite eluate prepared from solubilized liver extract<sup>†</sup> by adsorption at pH 3 and elution with 5% ammonia.

These results extend our knowledge of the rôle of inositol in the nutrition of the mouse, rat, and yeast to that of the chick as well. No pathological symptoms other than lack of growth have been observed. However, it is quite probable that our rations are not completely devoid of inositol and the studies are being continued in an attempt to obtain more satisfactory rations.

<sup>6</sup> Hegsted, D. M., Oleson, J. J., Elvehjem, C. A., and Hart, E. B., *Poul. Sci.*, 1940, **19**, 167.

<sup>7</sup> Hegsted, D. M., Oleson, J. J., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Nutr.*, 1940, **20**, 599.

<sup>†</sup> Wilson Laboratories.



## Augmentative Effects of Estrogens and Chorionic Gonadotropin.\*

C. F. FLUHMAN.

*From the Department of Obstetrics and Gynecology, Stanford University School of Medicine.*

It has been shown<sup>1, 2, 3</sup> that stilboestrol and estradiol dipropionate are able to stimulate increases in the ovarian weights of hypophysectomized rats, and that the concomitant administration of these substances with chorionic or equine hormone greatly enhances the gonadotropic effect. The present study demonstrates that a similar result may be obtained when estrogen is given to normal immature female rats prior to the injection of the chorionic gonadotropin.

A total of 93 immature female rats, 21 to 23 days of age, were used in this study. In each case the estrogen employed was dissolved in sesame oil and given subcutaneously in 0.1 cc dosages 3 times daily for 2 days, while the controls received the same amount of the plain oil. On the 3rd and 4th days the animals were given 3 daily subcutaneous injections of chorionic hormone in the amounts indicated in Table I. In one experiment A.P.L. (Ayerst, McKenna and Harrison) was used, and in the others an estrogen-free preparation (P.B.E.) made from blood of pregnant women as previously described.<sup>4</sup> The rats were autopsied on the 7th day. One group of 15 animals was given estrogen for 2 days in the manner described above, and was sacrificed on the 5th day without receiving the chorionic hormone.

The results are given in Table I. The injection of stilboestrol or alpha-estradiol dipropionate alone caused a slight increase in the weight of the ovaries, but this effect was not obtained with 6000 I.U. of a preparation of estrone (Theelin—Parke, Davis & Co.). A significant increase in the ovarian weights also was found when stilboestrol or estradiol dipropionate was given in high dosages previous to the administration of the chorionic hormone. This effect

\* Supported in part by the Rockefeller Fluid Research Fund of Stanford University School of Medicine.

<sup>1</sup> Williams, P. C., *Nature*, 1940, **145**, 388.

<sup>2</sup> Pencharz, R. I., *Science*, 1940, **91**, 554.

<sup>3</sup> Simpson, M. E., Evans, H. M., Fraenkel-Conrat, H. L., and Choh Hao Li, *Endocrinol.*, 1941, **28**, 37.

<sup>4</sup> Fluhmann, C. F., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 1193.

TABLE I.

Estrogen or control substance			Chorionic hormone	No. of rats	Avg body wt	Uterus	Ovaries
Sesame oil	0.6 cc		A.P.L. 750 I.U.	6	41	.150	.034
Di-ovocycin*	0.12 mg		"	6	39	.115	.048
"	1.5 "		"	6	39	.121	.067
Amniotin†	1200 I.U.		"	6	45	.144	.040
Sesame oil	0.6 cc		P.B.E. 1.5 cc	5	50	.083	.016
Stilboestrol	1.5 mg		"	5	50	.080	.034
"	3.0 "		"	5	43	.084	.049
Sesame oil	0.6 cc		P.B.E. 1.5 cc	6	38	.067	.016
Stilboestrol	3.0 mg		"	6	42	.084	.044
"	6.0 "		"	6	43	.096	.057
"	9.0 "		"	6	38	.090	.045
Sesame oil	0.6 cc		P.B.E. 1.5 cc	5	51	.078	.021
Theelin‡	1200 I.U.		"	5	48	.073	.027
"	6000 "		"	5	49	.087	.023
Di-ovocycin	1.5 mg		none	5	44	.119	.022
Theelin	6000 I.U.		"	5	38	.086	.013
Stilboestrol	9.0 mg		"	5	40	.117	.021
Uninjected controls				6	40	.037	.013

\*Alpha-estradiol dipropionate (Ciba).

†Amniotin (Squibb); estrogenic substances.

‡Theelin (Parke, Davis); estrone.

I.U. refers to international unit of estrone.

is similar to that obtained with testosterone propionate,<sup>5</sup> and may point to an important difference between various estrogens since the increases with amniotin and theelin were comparatively slight.

*Summary.* The preliminary administration to immature rats of high dosages of stilboestrol or alpha-estradiol dipropionate produces a significant increase in the effect of chorionic hormone on the resultant ovarian weight.

<sup>5</sup> Fluhmann, C. F., *Endocrinol.*, 1941, **28**, 214.

### Estrogen Content of the Blood at Four-Hour Intervals.\*

AMERICO S. ALBRIEUX.<sup>†</sup> (Introduced by C. F. Fluhmann.)

*From the Department of Obstetrics and Gynecology, Stanford University School of Medicine.*

Several investigators have demonstrated that there is a periodic variation in the estrogen content of the blood of women at different stages of the menstrual cycle (Frank and Goldberger, Siebke, Mazer and Goldstein, Fluhmann and others). The methods of bioassay used by these authors, however, require such large amounts of blood that it has not been possible to use them for determining whether or not the concentration of the hormone remains constant during the course of each day.

The test employed in this study requires only a few cubic centimeters of whole blood and is based on the intravaginal application method first suggested by Berger<sup>1</sup> and Lyons and Templeton.<sup>2, ‡</sup> Complete details of the procedure are reserved for a future communication, but it consists essentially of the introduction of small compressed pellets (40 to 80 mg) of whole blood, previously dried by dialysis,<sup>3</sup> into the vagina of standardized adult spayed rats. Three animals were used for each dose level and the results were judged from vaginal smears made at 48 and 60 hours following the introduction of the pellets. The results are given in terms of biologic rat units.

This report is based on 4 subjects, one of whom was examined at 2 different stages of the menstrual cycle. Two were normal women, one complained of polymenorrhea, and the fourth had been operated upon 11 days before for an ectopic gestation. In each instance 5 specimens of blood were obtained at 4-hour intervals during the course of a 24-hour period and the estrogenic hormone content determined.

The results are given in Table I, and it is seen that although the

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\*Supported in part by the Rockefeller Fluid Research Fund of Stanford University School of Medicine.

<sup>†</sup> John Simon Guggenheim Fellow.

<sup>1</sup> Berger, M., *Klin. Wchnschr.*, 1935, **14**, 1601.

<sup>2</sup> Lyons, W. R., and Templeton, H. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1936, **33**, 587.

<sup>‡</sup> In our hands this method has proved sufficiently delicate to respond to one-tenth of the international standard of estrone dissolved in sesame oil.

<sup>3</sup> Thalhimer, W., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **37**, 639.

TABLE I.

Patient	Day of cycle	Blood estrogen in rat units per 100 cc whole blood				
		8 a.m.	12 m.	4 p.m.	8 p.m.	Midnight
G	26	350	500	320	500	300
A	23	250	250	250	300	250
K	1	250	250	250	250	250
"	16	350	300	300	300	300
S	*	300	300	300	350	300

\*Eleven days after operation for ectopic gestation.

amount of estrogen varied during the course of the day in 4 out of 5 cases, the difference may be considered as significant (over 150 rat units per 100 cc of whole blood) in only one instance. A difference of 50 units was observed on 3 occasions but it is doubtful that this change is of importance. There was no consistency in the time of day or night at which the rises occurred.

*Summary.* The estrogenic hormone content of the blood of women was determined at 4-hour intervals during the course of one day in 5 instances. A significant diurnal rise was noted once, a minor increase in 3 cases, and a constant level was found in one case.

13147

### Concentration of Estrogenic Hormones in Blood Serum and Blood Cells.\*

AMERICO S. ALBRIEUX.<sup>†</sup> (Introduced by C. F. Fluhmann.)

*From the Department of Obstetrics and Gynecology, Stanford University School of Medicine.*

Since the work of Kemp and Bjergaard<sup>1</sup> it has been accepted that the estrogens in the blood of pregnant women are about equally divided between the plasma and the cells. These assays, however, were conducted following chemical extraction, and a very different result has been found in non-pregnant individuals by using dried blood for direct examination.

In this study the serum and blood cells were separated by centrifugation and then dried by placing in cellophane tubes on which an

\* Supported in part by the Rockefeller Fluid Research Fund of Stanford University School of Medicine.

<sup>†</sup> John Simon Guggenheim Fellow.

<sup>1</sup> Kemp, T., and Bjergaard, K. P., *Compt. rend. Soc. de Biol.*, 1932, **111**, 329.

TABLE I.

Specimen	Diagnosis	Day of cycle	Rat units of estrogen per 100 cc	
			Serum	Blood cells
B 3332	Dysmenorrhea	20	200	500
B 3343	"	2	200	400
B 3355	"	9	300	500
B 3367	"	16	300	600
B 3371	"	11	220	900
B 3384	"	21	250	400
B 3393	"	1	200	700
B 3406	"	12	220	500
B 3409	Sterility	13	220	500
	"	21	200	400
	"	28	200	400
	Polymenorrhea	11	180	400
	"	18	250	450
	"	25	200	400
B 3303	Amenorrhea		200	500
B 3317	"		280	600
B 3335	"		250	600
B 3307	Menopause		200	500
B 3316	"		300	600
B 3327	"		150	400

electric fan was allowed to play for a few hours.<sup>2</sup> The resultant powders were then compressed into small pellets which could be inserted directly into the vaginae of standardized adult spayed rats. Three animals were used for each dose level, and the results were based on vaginal smears made 48 and 60 hours after the introduction of the pellets.

The results of 20 observations are given in Table I, which shows that when bioassays are made in this manner the cells contain from 2 to 4 times as much estrogen per unit volume as the blood serum.

*Summary.* The blood serum and blood cells of a group of 20 non-pregnant women were examined for their estrogenic hormone content by the intravaginal application of pellets made from desiccated material. The blood cells were found to contain two or more times as many rat units per unit volume as the blood serum.

<sup>2</sup> Thalhimier, W., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **37**, 639.



## Confirmation *in vitro* of Griffith-Campbell Method for Measuring Blood Volume.

J. Q. GRIFFITH, JR.\*

*From the Robinette Foundation and the Medical Clinic of the Hospital, University of Pennsylvania.*

Beckwith and Chanutin<sup>1</sup> state that "in the hypertensive partially nephrectomized rat, the plasma volume was increased, the red cell volume decreased and the total blood volume remained unchanged." In referring to a paper by Griffith and Ingle,<sup>2</sup> who found an elevated blood volume in hypertensive partially nephrectomized rats, they say, "On inspection of their procedure for estimating blood volume, it appears that they neglected to take the red cell volume into consideration and the values reported by them probably represent plasma volumes . . . the increased plasma volumes (found by Beckwith and Chanutin) . . . agree with the reinterpreted data of Griffith and Ingle."

The blood volume method used by Griffith and Ingle was that previously reported by Griffith and Campbell.<sup>3</sup> In this method 0.3 cc of 5% vital red is injected intravenously and 4½ minutes later 50 cmm of whole blood are removed and placed in 2.95 cc of physiologic saline, the resulting volume being 3 cc and the blood dilution 1:60. After centrifuging, 2 cc of the supernatant fluid are removed and compared in a colorimeter with suitable standards. The red cells remain in the 1.0 cc left in the test tube, and a slight error is thus introduced in that the volume which the cells occupy is included in the 3 cc but does not contain an aliquot portion of the dye. The dye is actually contained in 3 cc less the volume of the cells. If the cells comprised 50% of the total blood, the figure would be 3.0 cc less .025 cc or 2.975 cc. This represents an error of 0.8%, and would make the calculated figure too low by that amount. However, the accuracy of the method is probably only about 5%, and such a correction appears unnecessary. Moreover, the range of variable concentrations of red cells consistent with life is such that the highest

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\*Atwater Kent Fellow in Medicine.

<sup>1</sup> Beckwith, J. R., and Chanutin, A., *PROC. SOC. EXP. BIOL. AND MED.*, 1941, **46**, 66.

<sup>2</sup> Griffith, J. Q., and Ingle, D. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **44**, 538.

<sup>3</sup> Griffith, J. Q., and Campbell, R., *PROC. SOC. EXP. BIOL. AND MED.*, 1937, **36**, 38.

red cell volume would differ from the lowest by somewhat less than 25 cmm. In comparisons of blood volumes for different animals the actual error from omitting this correction would probably be less than 0.5%.

To confirm this *in vitro*, the following experiment was performed: (1) To 5 cc of citrated blood having an hematocrit value of 43, 0.3 cc of 5% vital red was added and, after thorough mixing, 50 cmm of the citrated blood with the dye were removed and carried through the procedure detailed above. The value was found to be 4.9 cc. (2) Five cc of citrated blood mixed with serum to give an hematocrit value of 18 was carried through the same procedure. The value found was 4.7 cc. (3) Five cc of serum were treated similarly. The value found was 5.0 cc.

Thus the differences between the results of Beckwith and Chanutin and those obtained by Griffith and Ingle cannot be explained by the latter's misconception of their blood volume method. Certain further points should also be kept in mind: (1) Chronicity of the experiment. Beckwith and Chanutin used animals operated 3 to 4 months previously. Blood pressures were not measured but hypertension was "judged by the tortuosity and dilatation of the carotids." Griffith and Ingle used animals approximately 2 weeks after the second operation or 3 weeks after the first. They actually measured blood pressure, and not all animals were hypertensive. Tortuosity and dilatation of the carotids were not noted. (2) The presence or absence of anemia. Beckwith and Chanutin's rats were anemic. Griffith and Ingle did not measure the red cell volume in the animals whose blood volume was measured, but similar animals did not show a significant degree of anemia as compared with controls. If a normal red cell volume were added to the plasma volume figures of Beckwith and Chanutin, the results for blood volume would approach those of Griffith and Ingle. (3) Technical differences. The two groups of workers used different dyes and different times for collecting samples. Griffith and Ingle removed less renal tissue in the second operation than did Beckwith and Chanutin and many of their animals recovered from their hypertension, whereas those of the latter apparently did not.

## 13149 P

Isolation of L Type Growth from a Strain of *Bacteroides funduliformis*.<sup>\*†</sup>

LOUIS DIENES.

*From the Department of Pathology and Bacteriology, Massachusetts General Hospital, and the R. W. Lovett Memorial, Harvard Medical School, Boston.*

It has been described in a previous note that in cultures of certain bacterial strains belonging to various species tiny colonies, similar to young colonies of the pleuropneumonia group of organisms develop.<sup>1</sup> During the last year these colonies were isolated in pure culture from a strain of *flavobacterium* and one of *Bacteroides funduliformis* respectively. The colonies isolated from the *flavobacterium* always died out after 2 or 3 transplants; those isolated from the *funduliformis* apparently can be indefinitely maintained in cultivation. Colonies resembling those of the organism of pleuropneumonia bovis were first isolated by Klieneberger from cultures of *Streptobacillus moniliformis*.<sup>2</sup> She designates them with the letter L. Recently she described the isolation of similar cultures from a Gram negative streptobacillus causing suppurative lesions in guinea pigs.<sup>3</sup> The characteristics which differentiate these L type colonies from the usual bacterial colonies are the following:

The colonies are very tiny. The growth extends not only on but beneath the surface of the medium. The growing elements of the colonies are tiny pleomorphic granules and fine filaments which swell up and may form large round bodies of 5 to 10 micra. The pleomorphism of the cultures is accentuated by the softness of the elements. They are disfigured by the slightest tearing and for this reason are not recognizable in dry smears. The colonies have a marked tendency to autolyze.

A strain of *funduliformis* was isolated from a case of generalized peritonitis. Immediately after isolation the bacterium was similar in many respects to *Streptobacillus moniliformis*. It grew in the form of Gram negative segmented filaments which in fully developed

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<sup>\*</sup> The expenses of this investigation were defrayed in part by a grant from the Commonwealth Fund.

<sup>†</sup> This is publication Number 53 of the R. W. Lovett Memorial, Harvard Medical School.

1 Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 636.

2 Klieneberger, E., *J. Path. and Bact.*, 1935, **40**, 93; 1936, **42**, 587.

3 Klieneberger, E., *J. Hygiene*, 1940, **40**, 204.

colonies became pleomorphic and many of the bacteria swelled up into large round bodies. The colonies autolyzed within a short time and after 3 days' incubation could not be further transplanted. The strain is strictly anaerobic and not pathogenic for mice or guinea pigs. The colonies grew to a larger size and the bacterial filaments were thicker than those of *Streptobacillus moniliformis*. L type growth never developed beneath the colonies.

The growth of this strain was most abundant on rabbit serum agar plates. When fresh growth from such plates was transferred to horse blood agar plates only a few bacterial colonies developed on the thickly inoculated places. After 2 days' incubation many very tiny colonies appeared between the bacterium colonies and on the less thickly inoculated parts of the plate. These tiny colonies in a stained agar preparation were indistinguishable in appearance from young colonies of the Ll of Klieneberger. They consisted of a few deeply stained large bodies lying on the surface of agar and of small pleomorphic deeply stained granules growing like roots in several directions into the agar. The technic used for the staining of the cultures has been described previously.<sup>4</sup>

Squares of agar containing the tiny colonies were cut out and transferred to boiled blood ascitic fluid or rabbit serum agar plates so that the colonies were lying on the fresh medium. No bacterial growth developed in the transplants but the original L type of colonies increased considerably in size and a similar growth developed beneath the colonies in the fresh medium. Proceeding in a similar way, in one series a good growth of L type of colonies was obtained in 12 consecutive transfers. If an agar block with well developed colonies was pushed over the surface of the medium a few colonies developed on the smeared area but thus far the cultures can be kept growing in successive transfers only beneath the agar blocks. The fully developed colonies as well as the young ones were similar to the Ll of Klieneberger. They consisted of similar elements and their surface was covered, as in the case of Ll colonies, with deeply stained large round bodies. It was noticeable, however, that the colonies were looser than the Ll colonies. They branched out more into the medium and continued to grow for a longer period. Like their parent bacterial strain the L type of culture obtained from *funduliformis* grew only under anaerobic conditions.

Another strain of *Bacteroides funduliformis* which was studied produced no L type colonies though the bacteria on certain media swelled up into large round bodies.

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<sup>4</sup> Dienes, L., *J. Inf. Dis.*, 1939, **65**, 24.

The isolation of the L type of growth was made possible both in the case of *flavobacterium* and of *funduliformis* by the elimination of viable bacteria by autolysis. The other cultures also in which the L type of colonies were observed, as in certain strains of *Bacillus coli*, *Bacillus influenzae* and of the gonococcus, possess a tendency to autolyze. In these cultures, however, the autolyzing was not complete and thus far all attempts to isolate the L type of growth have remained unsuccessful because the transplants were overgrown by bacteria.

## 13150

**Relative Germicidal Action of Some Halogenated Phenols and Their Phenolates.**

E. J. ORDAL. (Introduced by W. P. Larson.)

*From the Department of Bacteriology and Pathology, University of Washington, Seattle.*

Klarmann, Shternov and von Wowern<sup>1</sup> showed that the substitution of one, 2, and 3 atoms of chlorine into the phenol molecule led to a progressive increase in the germicidal action of the resulting compounds. Unfortunately, at the same time the solubility of the phenolic compounds decreased markedly with substitution. The sodium salts of these halogenated phenols possess a considerably greater solubility than the phenols themselves, but there is no explicit information available in the literature as to the actual germicidal power of the phenolates.

Tilley and Schaeffer<sup>2</sup> showed that the germicidal efficiency of phenol decreased with an increase in percentage of sodium hydroxide added until the percentage of sodium hydroxide equalled or slightly exceeded that required to neutralize the phenol. Phenol is a weak acid with the acid dissociation constant  $K'_a = 1.06 \times 10^{-10}$  at 25°C.<sup>3</sup> Consequently a completely neutralized solution of phenol will be strongly alkaline, and an attempt to evaluate the actual germicidal action of the phenolate is complicated by the alkalinity of the solution. The substitution of chlorine atoms into the phenol molecule increases

<sup>1</sup> Klarmann, E., Shternov, V. A., and von Wowern, J., *J. Bact.*, 1929, **17**, 423.

<sup>2</sup> Tilley, F. W., Schaeffer, J. M., *J. Agr. Res.*, 1931, **43**, 611.

<sup>3</sup> *International Critical Tables*, prepared by National Research Council of the U.S.A., 1929.



the strength of the molecule as an acid and makes it possible to evaluate the relative germicidal action of some undissociated phenols and their phenolates without undue interference by excess acidity or alkalinity.

In this investigation 3 substituted phenols: o-chlorophenol, 2, 4 dichlorophenol and 2, 4, 6 trichlorophenol were tested in moderately acid and alkaline solutions. The acid series of solutions were made by diluting weighed quantities of the several phenols with M/60 phosphate buffer of approximately pH 6.1. The alkaline solutions were made by adding standard sodium hydroxide to weighed samples of the phenols to adjust them to the neighborhood of pH 9.8 and diluting with M/60 carbonate buffer at pH 9.8. The pH of each solution tested was accurately determined with a glass electrode.

Germicidal tests were made using the Food and Drug Administration<sup>4</sup> procedure for *Staphylococcus aureus*, except that the temperature of the experiment was 25°C and that subcultures were made into molten agar at 42-43°C and plates poured instead of using tubes of broth. Colony counts were made after 48 hours' incubation at 37°C.

The relative germicidal effects in acid and alkaline solutions are shown in Table I.

TABLE I.  
Germicidal Action of Chloro-Substituted Phenols in Acid and Alkaline Solutions.

Compound	Cone. of phenol in g per L	pH of sol.	Colonies per plate		
			5 min	10 min	15 min
o-chlorophenol	4.00	6.09	0	0	0
	3.63	6.10	24	0	0
	3.33	6.10	320	37	4
	58.8	9.93	0	0	0
	39.2	9.89	20	0	0
	29.4	9.87	1100	670	380
2,4-dichlorophenol	0.833	6.10	3	0	0
	0.769	6.10	39	0	0
	0.715	6.10	344	25	0
	40.7	9.98	0	0	0
	30.5	9.93	12	0	0
	24.4	9.90	124	36	0
2,4,6-trichlorophenol	16.3	9.86	1300	700	370
	.571	5.84	0	0	0
	.500	5.86	45	0	0
	.444	5.88	450	58	6
	40.0	9.76	0	0	0
	34.3	9.80	14	3	0
	30.0	9.82	230	40	0

<sup>4</sup> Circular No. 198, 1931, U. S. D. A.

TABLE II.  
Composition of Critical\* Germicidal Solutions.

Compound	pK' <sub>a</sub> at 25°C	pH of sol. tested	Total phenol added in moles/L × 10 <sup>3</sup>	Cone. of undissociated phenol in moles/L × 10 <sup>3</sup>	Cone. of phenolate ion in moles/L × 10 <sup>3</sup>
o-chlorophenol	8.5	6.10	28.2	28.1	.1
		9.89	305.	11.9	293.1
2,4-dichlorophenol	7.7	6.10	4.72	4.62	.1
		9.93	187.3	1.2	186.1
2,4,6-trichlorophenol	6.2	5.86	2.53	1.68	.85
		9.80	173.6	.04	173.6

\*Solutions most nearly sterilizing in ten minutes but not in five at 25°C.

It is evident from Table I that there is a sharp drop in the germicidal activity when the solutions are made alkaline.

For purposes of comparison the solutions most nearly sterilizing in ten minutes but not in five were designated as critical solutions. The actual concentrations of undissociated phenols and of phenolates were calculated for these solutions from the total concentration of the phenol and from the pK'<sub>a</sub> values of the substituted phenols. The pK'<sub>a</sub> values for 2,4-dichlorophenol and 2,4,6-trichlorophenol were obtained from Krah1 and Clowes<sup>5</sup> while that for o-chlorophenol was obtained from Murray and Gordon.<sup>6</sup> The results are expressed in Table II.

It is evident from Table II that the germicidal strength of the phenols increases directly with the number of chlorine atoms substituted, thus confirming the results of Klarmann, Shternov and von Wowern.<sup>1</sup> Comparison of the acid and alkaline solutions show that the undissociated phenolic compounds are far more germicidal than their phenolates, although the phenolates are not without toxicity. In the case of 2,4,6-trichlorophenol it is possible to get an estimate of the relative germicidal strength of the undissociated compound as compared with the phenolate since only a trace of the undissociated phenol is to be found in the solution at pH 9.80. From the ratio of the concentration of phenolate at pH 9.80 to that of the phenol at pH 5.86 it appears that the undissociated phenol is approximately 100 times more active than its phenolate under the conditions of the experiment.

<sup>5</sup> Krah1, M. E., and Clowes, G. H. A., *J. Cell. and Comp. Physiol.*, 1938, **11**, 1.

<sup>6</sup> Murray, J. W., and Gordon, N. E., *J. Am. Chem. Soc.*, 1935, **57**, 110.

### Isolation of Uroporphyrin from the Feces in Idiopathic Porphyrin.\*

SAMUEL SCHWARTZ AND C. J. WATSON.

*From the Division of Internal Medicine, University of Minnesota Hospital, Minneapolis, Minn.*

Since the first description of uroporphyrin by H. Fischer<sup>1</sup> this substance has been believed to be excreted only in the urine. Numerous publications concerning the excretion of porphyrins under normal or pathological conditions have failed to mention the occurrence of uroporphyrin in the feces. We have found it in the form of the zinc complex in the feces of four out of five cases of idiopathic porphyria.<sup>†</sup> Its presence was questionable in the fifth. Although uroporphyrin was readily identified in the feces of cases 1 and 3, the method of purification available at the time these were studied was inadequate to permit crystallization. By means of an improved method entailing chromatographic analysis, crystalline uroporphyrin has been isolated from cases 4 and 5. The method employed was as follows: The untreated feces was ground in a mortar with methyl alcohol saturated in the cold with hydrochloric acid gas. The methyl alcohol extract was filtered from the fecal residue on a Buchner funnel, and the residue was then extracted twice more by grinding in a mortar with an additional amount of methyl alcohol HCl. The combined methyl alcohol extract was allowed to stand overnight. It was then mixed with chloroform and several volumes of water and shaken in a separatory funnel. The chloroform fraction was washed twice with water and once with 10% NH<sub>4</sub>OH. Shaking with an equal volume of 7% NaCl quickly breaks any emulsion. The chloroform was washed twice more with NaCl solution. It was next filtered through chloroform moistened paper and mixed with 10 volumes of petroleum ether. The filtrate was then passed through a column of Brockmann's Al<sub>2</sub>O<sub>3</sub> (Merck) 1.5 x 12 cm in dimensions. The precipitate was redissolved in the least possible concentration of chloroform in petroleum ether. This was also filtered through the

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\* Aided by a grant from the John and Mary R. Markle Foundation, New York City.

<sup>1</sup> Fischer, H., *Z. f. physiol. Chem.*, 1915, **95**, 34.

<sup>†</sup> We are indebted to Doctors A. R. Hall and John Noble, St. Paul, Minnesota, Doctors C. E. Lynn and A. G. Plankers, Dubuque, Iowa, and Dr. W. H. Ford, Minneapolis, Minnesota, for their permission to study cases 1, 2 and 3 respectively.

$\text{Al}_2\text{O}_3$  column after the original 1:10 mixture had almost completely passed through. A chromatogram as seen in Fig. 1 was then developed by elution with increasing concentrations of  $\text{CHCl}_3$  in petroleum ether.

The proportions of  $\text{CHCl}_3$  to petroleum ether as given for the various zones in Fig. 1, refer to the mixture used in eluting each zone. For example, the lower orange zone was completely removed with the 1:10 mixture while the uroporphyrin zone<sup>2</sup> was moved but slightly, and eventually required a 1:1 mixture for complete elution. Thus it is seen that the chromatogram in Fig. 1 is really composite since zones 1, 2, and 3 were removed before the remainder of the chromatogram was well developed.

It should be noted that a number of samples of feces from cases 4 and 5 were individually subjected to the above procedure. In some instances the total amount of pigment was relatively too large for the first  $\text{Al}_2\text{O}_3$  column with the result that there was some over-

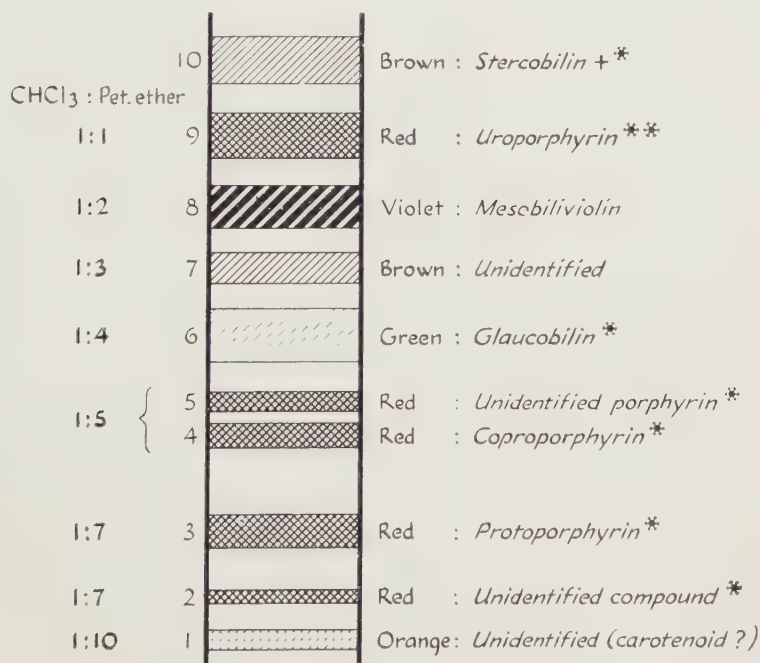


FIG. 1.

Chromatogram as developed by elution with increasing proportions of  $\text{CHCl}_3$  in petroleum ether.

\*Obtained in crystalline form from the feces of case 4. Present also in feces of case 5, but not crystallized.

\*\*Crystallized from feces of both cases 4 and 5 repeatedly.

<sup>2</sup> Watson, C. J., *J. Biol. Chem.*, 1936, **114**, 47.

lapping of zones and the primary chromatogram in these instances was not as well defined as shown in Fig. 1. This necessitated preparation of secondary chromatograms from 2 or more overlapping zones.

The eluent from each of the zones noted in Fig. 1 was concentrated on the water bath to a volume of 1-2 cc, and hot methyl alcohol was then added. After further concentration to remove any remaining petroleum ether or chloroform, crystallization occurred, on cooling, in the fractions as indicated by the asterisks in Fig. 1. The stercobilin from zone 10 was not crystallized as the ester, but rather as the hydrochloride after saponification by heating for one-half hour in dilute NaOH, followed by acidification with HCl, and further treatment in the usual manner.<sup>2</sup> The glaucobilin methyl ester from case 4 crystallized directly and after 2 recrystallizations melted at 222-223°C (uncorrected). The protoporphyrin methyl ester from case 4 melted at 216-217°C (uncorrected) after 3 crystallizations from  $\text{CHCl}_3$ -methyl alcohol. On one occasion coproporphyrin III methyl ester was obtained from case 4, the crystals melting at 160-165°C. It may be emphasized that the esters of uro-, copro-, and protoporphyrin have always been noted on the  $\text{Al}_2\text{O}_3$  column in the order shown in Fig. 1. Uroporphyrins were isolated repeatedly from both cases 4 and 5. These were spectroscopically identical with uroporphyrin as isolated from the urines of all 5 cases. The melting points of the methyl esters clearly indicated a mixture of the types I and III isomers. H. Fischer and Hofmann<sup>3</sup> have shown that uro- I ester melts at 302°C (uncorrected) while uro- III ester was found by Waldenström<sup>4</sup> to melt at 258°C (uncorrected). In the present study crystals from case 4 melted at 267°C (uncorrected), and from case 5 at 280°C (uncorrected). On decarboxylation of the uroporphyrins by the usual method<sup>5</sup> a mixture of coproporphyrins I and III was obtained in both cases. Those of case 4 were separated completely by the  $\text{Al}_2\text{O}_3$ -acetone method as previously described.<sup>6</sup> The copro- I ester melted at 248°C (uncorrected), and the copro- III ester at 145°C (uncorrected), and after crystallization, at 175-180°C (uncorrected). The amount of the latter was insufficient for recrystallization which may explain the somewhat elevated second melting point. The characteristic dimorphic melting of copro- III methyl ester was clearly evident, however. It may be

<sup>3</sup> Fischer, H., and Hofmann, H. J., *Z. f. physiol. Chem.*, 1937, **246**, 15.

<sup>4</sup> Waldenström, J., *Z. f. physiol. Chem.*, 1935, **233**, 1.

<sup>5</sup> Fischer, H., *Z. f. physiol. Chem.*, 1916, **97**, 109.

<sup>6</sup> Watson, C. J., and Schwartz, S., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 7.



noted further that crystals were of the typical rosette type, while those of the copro- I ester were the characteristic curved needles which were relatively insoluble in methyl alcohol.

Waldenström<sup>1</sup> found that uroporphyrin III is extracted by ethyl acetate from an aqueous solution at pH 3.0-3.2 (barely gray-blue to Congo paper by the addition of a very small amount of dilute HCl). The uroporphyrin III from each of the above cases behaved in this manner. It must be emphasized, however, that uroporphyrin is not unique among the porphyrins in being ether insoluble and ethyl acetate soluble, since we have encountered at least one and probably two porphyrins behaving in this manner, but nevertheless clearly distinct from uroporphyrin. These have not yet been identified and are being studied further at the present time.

## 13152 P

### A Simple Test for Urinary Porphobilinogen.\*

C. J. WATSON AND SAMUEL SCHWARTZ.

*From the Division of Internal Medicine, University of Minnesota Hospital,  
Minneapolis, Minn.*

The urines of patients suffering from idiopathic porphyria have often been noted to exhibit strong Ehrlich reactions, in many instances at least, not due to urobilinogen. Waldenström's studies<sup>1, 2</sup> clearly demonstrated that the chromogen responsible for the Ehrlich reaction in these urines is quite distinct from urobilinogen, which is of course, most often implicated in other pathological states. During the past 3 years we have had opportunity to investigate urine samples from 5 cases of so-called "acute" idiopathic porphyria.<sup>†</sup> In each instance the urine contained the zinc complex of uroporphyrin in considerable amount. The subject of zinc uroporphyrin as a disease entity will be considered in a separate communication. These urines also exhibited Ehrlich reactions in varying degree, at times very

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\* Aided by a grant from the John and Mary R. Markle Foundation, New York City.

<sup>1</sup> Waldenström, J., Studien über Porphyrie, *Act. Med. Scand. Suppl.*, 1937.

<sup>2</sup> Waldenström, J., and Vahlquist, B., *Z. f. physiol. Chem.*, 1939, **260**, 189.

<sup>†</sup> We are indebted to Doctors A. R. Hall and John Noble, St. Paul, Minnesota, Doctors C. E. Lynn and A. G. Plankers, Dubuque, Iowa, and Dr. W. H. Ford, Minneapolis, Minnesota, for their permission to study cases 1, 2 and 3 respectively.

intense. In the first 4 cases, the freshly voided urine was already red-brown in color, becoming darker on exposure to light. In the fifth case, the fresh urine was for the most part normal in color, exhibiting a strong Ehrlich reaction. On standing in the light for several days the urine from this case likewise became a deep reddish-brown, containing now both the zinc complex of uroporphyrin and the reddish-brown porphobilin.<sup>2</sup> Waldenström pointed out that porphobilinogen is much less soluble in organic solvents than is urobilinogen.<sup>1, 2</sup> In addition to confirming this, we have found that the porphobilinogen aldehyde compound as formed in the Ehrlich reaction is wholly insoluble in chloroform, while that of urobilinogen is readily and easily extracted with this solvent. The reaction as we have carried it out is as follows: Equal parts of urine and Ehrlich's reagent<sup>‡</sup> are mixed in a test tube. To this mixture is then added an equal volume of a saturated aqueous solution of sodium acetate. A few cc of chloroform are then added, and the solutions are thoroughly mixed. The aldehyde compound of porphobilinogen remains in the aqueous fraction while that of urobilinogen is completely extracted by the chloroform. We have repeatedly convinced ourselves that a mixture of the two substances can be separated completely by this method. It should be emphasized that the solution must contain the concentration of salt as achieved by the above proportions; otherwise the extraction of urobilinogen will not be complete. Indol added to the urine exhibits a positive Ehrlich reaction with a color very similar to that given by either urobilinogen or porphobilinogen. The characteristic absorption band is found in the region of 570  $m\mu$  (max.) while that of the urobilinogen or porphobilinogen aldehyde compound is maximum at 562  $m\mu$ . The indol aldehyde color, like that of urobilinogen, is completely extracted by chloroform under the conditions noted. Thus it is believed that a chloroform insoluble compound, as obtained in the above method, is due to porphobilinogen and is pathognomonic of porphyria. The urine should be examined for this reaction in any case having symptoms suggestive of porphyria, such as unexplained abdominal pain, flaccid paralyses, or other nervous manifestations. Obviously the reaction will be of most value in the cases in which the freshly voided urine is normal in color. The relative insolubility of porphobilinogen in organic solvents has so far prevented purification of the substance, and it is not yet known whether porphobilinogen is also excreted as the zinc complex or whether the zinc is combined only with uroporphyrin.

<sup>‡</sup> 0.7 g p-dimethylaminobenzaldehyde; 150 cc concentrated HCl; 100 cc distilled water.

## 13153

**Effect of Sulfonamide Compounds upon Growth of Staphylococci in Presence and Absence of p-Aminobenzoic Acid.\***

WESLEY W. SPINK AND JEAN JERMSTA.

*From the Division of Internal Medicine, University of Minnesota Medical School, and University Hospital, Minneapolis.*

A series of observations has been made concerning the effect of sulfonamide compounds upon the growth of staphylococci. Comparative studies were made with sulfanilamide, sulfapyridine, sulfathiazole and sulfadiazine. At the same time, the inhibitory effect of p-aminobenzoic acid upon the bacteriostatic action of these compounds was investigated.

*Methods.* Four strains of *S. aureus* were utilized. They were isolated from patients having severe staphylococcal infections. Each strain produced coagulase and fermented mannite. To facilitate standard conditions, a synthetic medium was used throughout these studies. This chemically-defined medium was the same as described by Gladstone.<sup>1</sup> The advantages of using such a medium for studying the growth-inhibitory effects of the sulfonamide compounds are: it contains neither peptone nor neopeptone; it provides a basal medium of known constituency; and it supports growth of the organisms as readily as the usual broth media used for staphylococci.

Several generations of each of the 4 cultures were grown in the synthetic medium before utilizing a 24-hour culture in the experiments to be described. After several preliminary studies, it was found satisfactory for our present purposes to add 0.05 cc of a 1:10 dilution of the 24-hour culture to 11 cc of the basal medium. The approximate number of cocci added was determined by making agar pour plates of varying dilutions of the original inoculum. The following study was made with each of the strains. Control tubes contained only the basal medium and added organisms. Seven tubes containing the basal medium and organisms had p-aminobenzoic acid in varying concentrations added to them. The final concentration of p-aminobenzoic acid was respectively 2.0, 0.4, 0.08, 0.016, 0.0032, 0.00064, 0.000128 mg per 100 cc. The purpose of this was to determine whether p-aminobenzoic acid inhibited or stimulated the growth

\* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

<sup>1</sup> Gladstone, G. P., *Brit. J. Exp. Path.*, 1937, **18**, 322.

of staphylococci. Different concentrations of each of the sulfonamide compounds were added to tubes containing the basal medium and organisms. Freshly prepared solutions were made by dissolving the compounds in sterile distilled water. The sodium salts of sulfapyridine, sulfathiazole and sulfadiazine were used.<sup>†</sup> Under these conditions, the final concentrations of each of the sulfonamide compounds were 10, 5, 3, and 1 mg per 100 cc. Finally, a series of test tubes was set up containing the basal medium, organisms and different concentrations of p-aminobenzoic acid and each of the sulfonamide compounds as shown in Table I. All of the tubes with their contents were incubated at 37°C for 24 hours. This time interval was selected as being optimal for studying growth after many observations had been made using intervals of 12, 48, and 72 hours. The degree of bacterial growth was measured according to the turbidity of the contents in each tube at the end of 24 hours. For this purpose, readings were made with the Evelyn photoelectric colorimeter using a 540 filter. The contents of each tube were thoroughly mixed, transferred to previously standardized absorption tubes, and the amount of light transmitted through each tube was determined.

As a standard, a tube containing the water-clear basal medium was used. It was found that this method for determining bacterial growth was as satisfactory as ascertaining the number of viable organisms present by the pour-plate technic. The galvanometer readings, which are a logarithmic expression of light transmitted, were converted by means of logarithms to the per cent of light transmitted. Thus the greater the turbidity of the medium, the smaller the percent of light transmitted.

*Results.* Table I shows the results obtained with one of the 4 strains studied. Observations made with the other 3 strains gave essentially the same results. It is to be noted that different concentrations of p-aminobenzoic acid neither stimulated nor inhibited the growth of staphylococci. This is in agreement with the results of Strauss, Lowell and Finland, who studied the pneumococcus.<sup>2</sup> Woods, on the other hand, stated that p-aminobenzoic acid was an essential growth factor for *Streptococcus pyogenes*.<sup>3</sup> The effect of varying concentrations of each of the sulfonamide compounds upon bacterial growth is of interest. When the concentrations of each of the drugs was 10 mg per 100 cc growth of the organisms was markedly inhibited. When a concentration of 5 mg was

<sup>†</sup> The sodium sulfadiazine was supplied to us by the Lederle Laboratories, Pearl River, New York.

<sup>2</sup> Strauss, E., Lowell, F. C., and Finland, M., *J. Clin. Invest.*, 1941, **20**, 189.

<sup>3</sup> Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

TABLE I.  
Bacteriostatic Effect of Sulfonamide Compounds on *Staphylococcus aureus* in the Presence and Absence of p-Aminobenzoic Acid.

Growth expressed in % of light transmitted through culture									
Basal medium plus p-aminobenzoic acid	Basal medium plus sulfonamide compound	Basal medium + sulfonamide compound + p-aminobenzoic acid					Mg per 100 cc sulfonamide compound	Sulfonamide compound	
		2.0	.4	.08	.016	.0032	.00064	.000128	
28	28	81		28	28	35	72	83	10
28	28	86		28	32	73	86	87	
28	28	92	28	31	65	92			
28	28	93	29	81	91	93			5
28	28	50		28	28	29	39	51	
28	28	82		28	29	47	79	85	
28	28	91	28	31	39	87			3
28	28	93	28	39	93	92			
28	28	32		28	28	29	35	36	
28	28	72		28	30	31	56	72	1
28	28	91	28	28	32	76			
28	28	92	28	32	85	93			
28	28	29		28	28	28	29	29	
28	28	31		28	28	31	30	32	
28	28	53	28	28	29	33			
28	28	91	29	29	33	85			

Inoculum = 700,000 organisms per cubic centimeter.



present, the inhibitory action of sulfanilamide and sulfapyridine was considerably less than that obtained with sulfadiazine and sulfathiazole. This was also true with concentrations of 3 mg per 100 cc. The results were quite striking when a concentration of 1 mg per 100 cc was employed. Sulfathiazole was definitely more inhibitory than sulfadiazine, while this concentration of sulfanilamide and sulfapyridine had very little effect.

The inhibitory effect of p-aminobenzoic acid upon the bacteriostatic action of the compounds revealed that the larger quantities of the acid produced the greater inhibitory effect. Sulfathiazole inhibited bacterial growth more consistently than the other compounds, and p-aminobenzoic acid was the least effective in inhibiting this bacteriostatic action of sulfathiazole. In other words, these results show that the greater the inhibitory effect of a compound upon bacterial growth, the less the effect of p-aminobenzoic acid upon this action of the compound, which confirms the observations of others for the pneumococcus.<sup>2</sup> One observation that we have made several times was that the smaller concentrations of p-aminobenzoic acid in combination with a sulfonamide compound often appeared to inhibit growth more than was obtained with the sulfonamide compound alone. Control tubes showed that these smaller concentrations of p-aminobenzoic acid did not stimulate growth. These observations have been too few and inconsistent from which to draw final conclusions, but this paradoxical finding merits further investigation.

*Conclusions.* A synthetic, chemically-defined medium has proved satisfactory for the study of the bacteriostatic effect of the sulfonamide compounds upon staphylococci. Sulfathiazole and sulfadiazine have a definitely greater inhibitory effect upon growth compared to sulfapyridine and sulfanilamide, with sulfathiazole superior to sulfadiazine. While p-aminobenzoic acid inhibits this bacteriostatic action of all the compounds studied, the greater the effect of a compound upon growth of Staphylococci, the less inhibitory is p-aminobenzoic acid against this action of the compound.

## 13154 P

## Effect upon the Biliary Tract of Sectioning the Splanchnic Nerves.

FRANK E. JOHNSON. (Introduced by E. A. Boyden.)

*From the Institute of Anatomy, University of Minnesota.*

Recently the first case of sectioning the nerves to the choledochoduodenal junction in a patient was reported.<sup>1</sup> The reasons for believing that this procedure would not prove effective in relaxing the human sphincter of Oddi have already been set forth.<sup>2</sup> To test this more thoroughly, selection has been made of a laboratory animal in which inhibitory reflexes from the gut tract to the gall bladder have already been demonstrated<sup>3</sup> and in which the detailed distribution of nerves to the choledochoduodenal junction has been worked out<sup>4</sup>—namely the cat.

At laparotomy, the following procedures were carried out upon approximately 30 cats: (1) various autonomic nerves in the abdomen were severed (or doubly ligated); (2) insulated electrodes were sewed to the caecum by a flap of the rubber tube through which the enamelled wires were brought to the laparotomy wound; (3) the gall bladder was filled with lipiodol (Whitaker method). Some 15 hours later, by which time the animals had fully recovered, the latter were fed a mixture of egg yolk and milk by tube and then x-rayed at appropriate intervals with the object of ascertaining the rate of emptying of the gall bladder and the presence of inhibitory reflexes emanating from faradically induced contraction of the caecum.

Severance of the gastroduodenal nerve and plexus—specific nerves in the lesser omentum leading to the choledochoduodenal junction—resulted, in 8 animals, in retarding the evacuation of lipiodol. (The studies of DuBois and Hunt<sup>5</sup> were used as controls); and interruption of these nerves did not abolish the inhibitory reflex from the caecum to the gall bladder. These experiments, therefore, fail to support the view that such operations in man would relieve a spasm of the sphincter.

Severance of one or both splanchnic nerves, however, markedly accelerated the rate of emptying of the gall bladder (8 animals)

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<sup>1</sup> Reich, Henry, *Surg., Gynec. and Obst.*, 1940, **71**, 39.

<sup>2</sup> Boyden, E. A., *Surgery*, 1941, **9**, 443.

<sup>3</sup> Birch, C. L., and Boyden, E. A., *Am. J. Physiol.*, 1930, **92**, 301.

<sup>4</sup> Schulze, J. W., and Boyden, E. A., *Anat. Rec.*, 1941 (suppl. 2), **79**, 77.

<sup>5</sup> Du Bois, F. L., and Hunt, E. A., *Anat. Rec.*, 1932, **54**, 289.

and when both were severed the inhibitory reflex was abolished as well as the sensation of pain arising from a spastic caecum.

Various modifications of these experiments indicate that the 2nd lumbar roots of the coeliac ganglia as well as the lumbar sympathetic trunks are not pathways primarily involved in regulation of the biliary tract. Severance of the coeliac branch of the right vagus, in one instance, was followed by retardation of emptying.

The implications of these experiments are that evacuation of the extra hepatic biliary tract is inhibited by the sympathetic nervous system as mediated through the splanchnic nerves, and that the reason, perhaps, why severance of the specific nerves to the cholecho-duodenal junction does not relax this junction is that such nerves carry both vagus and sympathetic fibers and also innervate both the sphincter of Oddi and the contiguous portion of the duodenum.

### 13155 P

#### Bacteriostatic Effect of Paranitrobenzoic Acid on Pneumococci *in Vitro*.\*

JOSEPH T. KING AND AUSTIN F. HENSCHEL.

*From the Department of Physiology, University of Minnesota, Minneapolis, Minn.*

Mayer and Oechslin<sup>1</sup> reported that paranitrobenzoic acid and certain derivatives were effective in pneumococcal infections in mice although they showed little activity *in vitro*.

Gruhzit<sup>2</sup> found that paranitrobenzoate protected mice infected with *Streptococcus viridans*. He found little activity against beta hemolytic streptococci and none against Type I pneumococci. Miller<sup>3</sup> recently reported that *Streptococcus viridans* is inhibited *in vitro* by sodium paranitrobenzoate.

Barlow<sup>4</sup> finds the acid and ester effective in pneumococcal infections in mice.

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\* Aided by grants from the Medical Research Fund, Graduate School, the University of Minnesota, and the Department of Medical Research, Winthrop Chemical Co., Inc. Assistance in the preparation of these materials was furnished by the Work Projects Administration, official project No. 65-1-71-140, sub-project No. 237, and by the National Youth Administration. Drugs supplied by the Winthrop Chemical Co., Inc.

<sup>1</sup> Mayer, R. L., and Oechslin, C., *Compt. Rend. Soc. de Biol.*, 1939, **130**, 211.

<sup>2</sup> Gruhzit, O. M., *Arch. Path.*, 1940, **29**, 732.

<sup>3</sup> Miller, J. K., *J. Pharm. and Exp. Therap.*, 1941, **71**, 14.

<sup>4</sup> Barlow, O. M., unpublished data.

We have studied the effect of the acid and sodium salt on pneumococci in the tissue culture medium and with the methods which we used earlier in studies on Streptococci.<sup>5-9</sup>

Pneumococcus cultures were grown in rabbit serum extract of 7-8-day chick embryos. Cultures were incubated at 37.5°C for approximately 5 hours.

Drugs were dissolved in Tyrode's solution and sterilized by Berkefeld filtration. Stock solutions of sodium salts were 400 mg % concentration; the acid 40 mg %.

Dilution of the bacterial culture was made rapidly through Tyrode into the serum extract. The usual dilution was 10<sup>-8</sup> or 10<sup>-9</sup>.

The drugs were then added after pH adjustment (except as noted below). Buffer standards were checked with the glass electrode. The colorimetric method was used in making the adjustments. An appropriate amount of Tyrode was added to the control medium to compensate the dilution caused by the drug-Tyrode mixture. When drug-Tyrode mixtures of different concentrations were used, the stronger mixture was diluted to the strength of the weaker by addition of Tyrode before adding to the culture medium.

The cultures were planted on 22 mm round cover slips. Each

TABLE I.  
Bacteriostatic Effect of Paranitrobenzoic Acid, and the Sodium Salt, Compared with Sodium Sulfathiazol; Type II Pneumococcus; 15 mg%.

Organisms per cc	Control	Thiazol	P-NO <sub>2</sub> BA	Inhibition %	
				Thiazol	P-NO <sub>2</sub> BA
958	23.9*	6.7	11.5	72.0	51.9
940	27.2	8.1	12.0	70.3	55.9
254	65.9	10.8	14.4	83.6	78.1
3090	17.6	8.2	9.3	53.4	47.2
342	42.4	11.7	12.8	72.4	69.8
234	162.1	95.8	94.0	40.9	42.0
402	109.2	49.9	57.5	54.3	47.3
1128	71.8	40.0	43.7	44.3	39.1
468	52.1	30.1	33.6	42.3	35.5
252	124.7	38.1	54.3	69.4	56.4
368	101.1	21.2	25.7	79.0	74.6
108	99.6	29.7	39.9	70.2	59.9

\*Eyepiece units.

5 King, J. T., Henschel, A. F., and Green, B. S., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **38**, 810.

6 King, J. T., Green, B. S., and Henschel, A. F., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **38**, 812.

7 King, J. T., and Henschel, A. F., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 208.

8 King, J. T., Henschel, A. F., and Green, B. S., *J. A. M. A.*, 1939, **113**, 1704.

9 King, J. T., and Henschel, A. F., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **44**, 268.

culture was made up of 1 drop of heparinized rabbit plasma and 3 drops of bacterial suspension. Clotting occurs promptly. Calibrated pipettes were used to ensure cultures of the same volume in each group. Cultures were incubated as Maximow double cover-slip preparations at 37.5°C. Details have been described in previous papers.

Readings were made at 22-24 hours. The diameter of the colonies was measured with ocular micrometer and mechanical stage at 60X. (114 eyepiece units equal to 1 mm).

Each series is composed of 18 cultures, 6 of each drug and 6 controls.

The results of 12 series on pneumococcus, Type II (Bieter), are given in Table I.

The average inhibition of colony diameter for the 12 series was 61.0% for sulfathiazol; 54.5% for paranitrobenzoic acid and the salt.

There is no significant reduction in the number of colonies with either drug.

The first 5 series were done with the acid; the last 7 with the sodium salt. In the first 2 series the pH of the Tyrode-drug mixture was not adjusted before adding to the culture medium. In the 3rd series the two drugs were adjusted to the same pH. In the 4th and 5th series the drugs were adjusted to the pH of the Tyrode added to the control. In the last 7 series the sodium salt of paranitrobenzoic acid was used. In these, both drugs and the Tyrode added to the control were adjusted to pH 7.6 before adding to the culture medium.

Since the sodium salt of sulfathiazol is more alkaline than the salt of paranitrobenzoic, we have used sterile 1 N HCl for adjusting the former and 0.1 N HCl for adjusting the latter in order to avoid unequal dilution of drug solutions. There was, however, a slightly greater dilution of the benzoic compound than of the thiazol. On the average 3 drops of acid were required to adjust 10 cc of thiazol and 5 drops to 10 cc of benzoic.

Studies in progress on higher and lower concentrations show increasing inhibitory effect with increasing concentration of both drugs.

These findings on Type II pneumococci are in contrast with those of Mayer and Oechsli on Type I where they obtained little effect *in vitro*.

*Summary.* Paranitrobenzoic acid and the salt compare favorably with sulfathiazol in their bacteriostatic power against Type II pneumococci *in vitro*.



## 13156

# A New Method of Assay of Chromatophorotropic Hormone by Means of Excised Lizard Skin.

CORINNE KEATY AND ALLAN J. STANLEY. (Introduced by Albert E. Casey.)

*From the Zoology Department, Louisiana State University.*

The principle of the method to be described for testing pituitary chromatophorotropic substances is based on the fact that the dermal melanophores of excised skin of *Anolis carolinensis* respond to pituitary chromatophorotropin<sup>1</sup> by dispersion of the melanosomes.

The use of hypophysectomized frogs and lizards and light adapted frogs involves several undesirable conditions. They are all time-consuming, and in the case of operated reptiles, endocrine imbalance, especially thyroid effects, are involved. The use of excised skin of *Anolis* as a test object for measuring intensity of effect, is very rapid, entails no difficult operative procedure, requires only small amounts of material, and eliminates the influences of other possible endocrine or neural disturbances. The test has been found to be reliable. Uniform responses are produced on skin of well-fed animals 100% of the time without difficulty. The complete dispersion of melanosomes is easily detected since the color change from green to brown is very striking.

Acetone-dried pituitaries of several vertebrates (man, beef and dog) have been tested and complete assay of pituitaries of *Rana catesbeiana* has been made by this method. It has been found that frog material is best employed in dilutions of 1 mg in 20 cc cold-blood Ringer's solution. Stronger concentrations react upon the skin too rapidly for accurate observations to be made. The solution

TABLE I.

Melanosome Dispersion-rate of Excised Skin of *Anolis carolinensis* in 0.25 cc of Solution of 1 mg of Frog Pituitaries in 20 cc Frog Ringer's, at Room Temperature. Two hundred twenty-eight observations. The time is in minutes.

Material	Fresh, unfiltered	Fresh, filtered	24 hr refrig. unfiltered	24 hr refrig. filtered	24 hr room temp. unfiltered	24 hr room temp. filtered
Skin of Back and Thigh.						
Summer pituitary	6:12.7	6:1.0	6:12.8	6:50.1	12:41.2	12:34.7
Spring pituitary	3: 8.3	3:5.4	2:41.9	2:27.1	11:32.6	11:48.0
Skin of Belly.						
Summer pituitary	8:20.5	8:5.0	8:18.7	8:21.1	15:41.3	14:52.1
Spring pituitary	4:27.8	4:0.2	4:14.2	4: 0.9	10:29.4	10:16.6

<sup>1</sup> Hadley, C. E., *J. Exp. Zool.*, 1931, **58**, 221.

is refrigerated 24 hours and then filtered. The filtrate is saved for the test. It has been found that the unfiltered solution shows partial inactivation of the hormone after 24 hours at room temperature. Complete inactivation is accomplished after 48 hours.

Lizards used for such tests should be well-fed and watered daily, since variations in reactions or complete unresponsiveness results when the animals are not fed and watered regularly. Animals kept in the laboratory throughout the winter have been used successfully.

It has been found that skin from the back or top of the thigh is best for the test, since the scales in these regions possess greater numbers of melanophores and are of uniform size. Skin from the belly reacts more slowly. After removal, the skin is placed on the surface of cold-blood Ringer's solution, and cut with sharp scissors into small pieces. Pieces from 2 to 4 mm square are large enough. After 20 minutes in Ringer's, the skin becomes uniformly green. Scales along the cut edges, or scales injured by manipulation with instruments generally remain brown and will not react to other stimulation.

After complete melanosome condensation has been effected, a single piece of the skin is placed on the surface of 0.25 cc of the filtrate on a clean depression slide. A hypodermic syringe is used for measuring the amount. The color change to brown is best observed with a binocular dissecting microscope. The speed of the reaction is conveniently used to measure potencies of solutions prepared as described above. Table I shows the melanosome dispersion rates obtained by this method for pituitaries of *Rana catesbeiana* during early spring and late summer at room temperature.

The *Anolis* skin has been found to react to acetone-dried pituitaries of man (negro and white), cattle and dog.

Preliminary tests have been made of temperature variation on the rate of the reaction. The indications are that the  $K_{10}$  is applicable to the reaction.

## 13157

## Experiments on Immunization of Human Beings Against Influenza A.\*

WALTER P. MARTIN AND MONROE D. EATON.

*From the Research Laboratory, California State Department of Public Health, Berkeley, California.*

Previous investigators have reported stimulation of virucidal antibodies<sup>1, 2</sup> and some evidence of protection against influenza<sup>3</sup> in persons inoculated subcutaneously with active virus of influenza A. Others using formalinized inactive preparations<sup>4, 5</sup> have demonstrated an increase in antibodies, but found no evidence of active immunity in the vaccinated groups.

Recently Horsfall and Lennette<sup>6</sup> prepared a complex formalinized vaccine by infecting chick embryos simultaneously with canine distemper and influenza A virus. In a recent influenzal outbreak in California, this vaccine, and another vaccine made from active influenzal virus were used prophylactically.<sup>†</sup> The active-virus vaccine was prepared in our laboratory by growing the virus of influenza A strain Melbourne in minced chick embryo suspended in saline. This material was centrifuged at 1000 rpm for 10 minutes and the supernatant fluid was stored at  $-60^{\circ}\text{C}$  to be used as vaccine when needed. The intranasal MLD for mice was approximately 0.05 cc of a dilution of 1:1000. In this paper, the active tissue-culture vaccine will be termed V-1 and Horsfall's complex inactive vaccine V-2.

*Method.* In 2 large institutions in Northern California, which we shall designate X and Y, a group of the personnel was vaccinated

\* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of the Rockefeller Foundation and in coöperation with the California State Department of Public Health.

1 Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1937, **65**, 251.

2 Stokes, J., Chenoweth, A., Waltz, A., Gladen, R., and Shaw, D., *J. Clin. Invest.*, 1937, **16**, 237.

3 Stokes, J., McGuinness, A., Langner, P., and Shaw, D., *Am. J. Med. Sci.*, 1937, **194**, 757.

4 Med. Research Council, Special Report No. 228, 1938, 141.

5 Taylor, R., and Dreguss, M., *Am. J. Hyg.*, 1940, **31**, 31.

6 Horsfall, F. L. Jr., Lennette, E. H., and Rickard, E. R., *Jour. Exp. Med.*, 1941, **73**, 335.

† The authors are indebted to Dr. L. L. Stanley and Dr. P. W. Day for collecting the clinical data reported in these studies. The complex vaccine was supplied by the International Health Division Laboratories in New York.

2 weeks before an outbreak of acute febrile respiratory disease designated by the clinicians as influenza. At institution X about half of the vaccinated group was composed of new entrants and the other half of persons who entered the institution approximately 16 months previously. At institution Y the vaccinated group was selected according to serial number. Between the vaccinated and control groups no differences in chances of exposure to infection within the institution could be found. Approximately equal numbers of persons received V-1 and V-2. Each of the vaccines was given subcutaneously as a single inoculation in doses of 1 cc. All cases of acute febrile respiratory diseases were recorded after the completion of the vaccinations and attempts were made to study a representative group of the cases by serological methods. Bloods were obtained in the acute and convalescent stages of the disease and tested for complement-fixing antibodies against influenza A, by the method previously described<sup>7</sup> which was a modification of that originated by Smith.<sup>8</sup> Blood specimens were also obtained before and after the epidemic from a group of vaccinated individuals and from a group of nonvaccinated persons taken at random.

*Results.* The cases were recorded from December 1 to December 28 at both institutions. The peak of the epidemic extended from December 1 to 12 at institution X, and from December 17 to 19 at Y. At the close of the period of observation, the records submitted to us revealed 395 clinical cases at institution X and 413 at Y. Serological studies indicated that at both institutions the principal causative agent was the virus of influenza A. Of 50 cases tested by complement-fixation at institution X, 32 were positive for influenza A and all but 6 showed a rise of fourfold or greater. At institution Y, 24 of 26 cases tested showed a rise of fourfold or greater. All acute bloods were obtained during the peak of the epidemic at institution Y, whereas at X the epidemic had no sharp

TABLE I.  
Summary of Incidence in Vaccinated and Control Groups.

	Institution X			Institution Y		
	Total in group	Total cases	Incidence %	Total in group	Total cases	Incidence %
Vaccine —1	193	16	8.3	169	10	5.9
Vaccine —2	223	12*	5.4*	244	18	7.4
Total vaccinated	416	28	6.7	413	28	6.8
Controls (not vaccinated)	4560	365	8.0	2,487	385	15.5

\*Not counting 2 cases with onsets 6 and 7 days after vaccination.

<sup>7</sup> Eaton, M. D., and Rickard, E. R., *Am. J. Hyg.*, 1941, **33**, 23.

<sup>8</sup> Smith, W., *Lancet*, 1936, **2**, 1256.

peak and a number of the bloods were obtained late from some cases which were probably not influenza.

Table I reveals the morbidity in 2 vaccinated and 2 control groups. At institution X the difference between the incidence of infection in the control and vaccinated groups was much less definite than at institution Y where the incidence in the control group was 15.5% as compared to 6.8% in the vaccinated group. On reviewing the data in Table II these conflicting results may be explained in part by the lapse of time between vaccination and the peak of the epidemic. At institution X most cases that had received V-2 were recorded between 9 and 13 days after vaccination. However, most of those who had received V-1 at this institution became ill 2 weeks or more after vaccination. At institution Y the individuals were all vaccinated on 2 different days and the lapse of time between the completion of vaccinations and the peak of the epidemic was about 2 weeks.

Complement-fixation tests on sera taken before and after the epidemic from a group of 92 unvaccinated individuals selected at random in institution X revealed an increase in complement-fixing antibodies to influenza A in 9.8% with history of illness and in 19.5% with no evidence of clinical infection. There were apparently a great many cases of subclinical infection. These results are in accord with those found by Horsfall, Hahn, and Rickard.<sup>9</sup>

Table III presents serological results in the vaccinated group at institution X. These individuals were bled 4 to 6 months before, and one month after vaccination. Histories revealed no evidence of acute febrile respiratory disease in the interval between bleedings.

TABLE II.  
Date of Onset of Cases in Relation to Date of Vaccination.

Date of illness December	Institution X					Institution Y				
	Non- vac. cases	V-1 cases		V-2 cases*		Non- vac. cases	V-1 cases		V-2 cases	
		Days after vac.		Days after vac.			Days after vac.		Days after vac.	
		9-13	14-42	9-13	14-21		9-13	14-25	9-13	14-25
1-4	110		8	6	8					
5-8	99	1	1	2	6	2				
9-12	75	1	1		14	1				
13-16	43		2	2	71		2	2		
17-20	22		1		222		3		9	
21-24	10		1		53		2		7	
25-28	6				11					

\*2 cases receiving V-2 with onsets less than 9 days after vaccination occurred at institution X.

<sup>9</sup> Horsfall, F. L., Hahn, R. G., and Rickard, E. R., *J. Clin. Invest.*, 1940, **19**, 379.



TABLE III.  
Serological Results in Vaccinated Group at Institution X.

Vaccine	Test*	Pre-vaccination titer	No. showing increase of titer (post-vaccination) of			
			Nil	2-fold	4-fold	8-fold or over
V-1	C.F.—PR8	0 to 4	22	2	0	0
"	C.F.—PR8	8 or over	10	6	1	0
"	Neutr. PR8	0 to 4	1	3	1	0
"	" PR8	8 or over	4	0	1	0
V-2	C.F.—PR8	0 to 4	37	0	19	4
"	C.F.—PR8	8 or over	1	0	0	0
"	Neutr. PR8	0 to 4	0	4	2	2
"	" PR8	8 or over	3	2	2	0

\*C.F.—Complement-fixation test with PR8 antigen from infected mouse-lung.  
Neutr.—Neutralization-test against 1000 intranasal MLD of the strain PR8.

V-1 produced a rise in complement-fixing antibodies in 9 out of 32 of the individuals studied. In 10 cases the pre- and post-vaccination bloods were tested for neutralizing antibodies against the strain PR8. There was a significant rise of fourfold in 2 of the group and a twofold rise in 3 others. A twofold increase in neutralizing antibodies was considered of doubtful significance.

V-2 produced a rise in complement-fixing antibodies in 23 out of 38 individuals tested. In 15 cases the pre- and post-vaccination bloods were studied for neutralizing antibodies against PR8 virus. In 6 individuals there was a significant rise in antibodies after vaccination. The 3 cases which showed no rise at all had a high pre-vaccination titer, which may explain the lack of antibody-response to the vaccine in these individuals.

At institution Y only post-vaccination bloods were obtained from 13 individuals who had received V-1 and 15 individuals who had received V-2. In the complement-fixation test the geometric mean of the titer was 1:11 after V-1 and 1:12.9 after V-2. In the neutralization-test against 100 MLD of PR8 virus, the mean titer was 1:7.5 after V-1 and 1:16.8 after V-2.

*Discussion.* The protection afforded by either vaccine at institution X is questionable, but at Y the difference in incidence of cases between the vaccinated and control groups is sufficiently great to be of some significance, especially since the incidence of influenza A was high at this institution. The failure of V-2 to protect at institution X could be attributed to the short lapse of time between vaccination and the peak of the epidemic. However, in the groups vaccinated with V-1 at both institutions, the onset of illness in most cases was more than 2 weeks after vaccination, yet this vaccine

apparently gave some immunity in the vaccinated group at institution Y, but none at X.

V-2 produced a slightly, but perhaps not significantly, greater antibody-response than did V-1, as judged by complement-fixation and neutralization-tests. It is quite probable that subclinical infections accounted for some of the increase in antibodies. It also appeared that the response in complement-fixing antibodies produced by the two vaccines was considerably less in degree than the response following infection.

*Summary.* Studies were conducted at two large institutions during an outbreak of influenza A which was proven by complement-fixation tests. Persons at both institutions received a living virus and complex formalinized vaccine previous to the outbreak. An epidemiological survey revealed some protection at one institution, but none at the other. There was no obvious difference in the prophylactic efficacy of the two vaccines in the relatively small group of 829 individuals studied.

### 13158

#### Reaction of the Rat Omentum to Injections of Particulate Matter.

RALPH N. BAILLIF. (Introduced by Albert E. Casey.)

*From the Department of Anatomy, School of Medicine, Louisiana State University, New Orleans.*

It has been known for many years that certain cells widely distributed throughout the body have a special property which enables them to collect and store foreign particulate matter. On the basis of this common property it is now rather generally believed that these cells should be considered as belonging to a single system, the reticulo-endothelium. The critical reviews of Maximow<sup>1</sup> and Jaffé<sup>2</sup> are the most useful of the numerous articles available concerning the distribution, morphology and reactive characteristics of this system.

In spite of the many studies carried out in this field, the special

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<sup>1</sup> Maximow, A. A., The macrophages or histiocytes, Section XIX in Cowdry's *Special Cytology*, 1932, p.711.

<sup>2</sup> Jaffé, R. H., The reticulo-endothelial system, Section XV in Downey's *Handbook of Hematology*, 1938, p. 974.

physiologic properties which enable certain cells to collect and store foreign particles are still incompletely understood. In the experiments herewith reported special emphasis was placed on the cellular changes which take place during this process. An effort was also made to differentiate between phagocytosis proper and simple transmission of particles by cells, and special attention was directed to changes which occur in the Golgi apparatus of cells engaged in phagocytosis.

*Materials and Methods.* Seventy-nine albino rats were injected intraperitoneally with trypan blue, lithium carmine, or India ink suspended in saline solution or distilled water, in dosages varying from 0.2 to 137 cc per animal. After completion of the experimental period the animals were sacrificed under ether anesthesia. Early phases of phagocytosis were most numerous and most readily studied in animals killed 18-24 hours after a single injection of 0.5 to 1.0 cc. Later phases were best studied in animals given daily injections varying from 0.2 to 2.1 cc over periods ranging from 5 to 75 days and killed 18-24 hours after the last injection.

The tissues to be examined were fixed in Bouin's or Regaud's solution, or in Ludford's modification of the Mann-Kopsch solution. They were then dehydrated in dioxan, embedded in bayberry-paraffin, and sectioned at 5  $\mu$ . Slides were stained in Regaud's hematoxylin, Heidenhain's azan, or hematoxylin-eosin.

*Observations and Discussion.* Within 18 hours after the first injection of the particulate material a marked histiocytic response appears in the omental substance. The most striking feature of the reaction is the mobilization of enormous numbers of histiocytes, which are rapidly transformed into macrophages and ingest the incoming pigment.

Particulate matter enters the omentum (1) by surface transmission and (2) via blood vessels.

(1) It has been shown that the majority of pigment granules enter the omentum by passing between the mesothelial cells, only a few passing directly through them. The transformation of the covering cells into macrophages has never been observed. As soon as any particles pass into the omentum the resting histiocytes mobilize and begin to migrate toward the surface, where they are soon transformed into active phagocytes which engulf and store the entering particles (Fig. 1).

(2) Within a few hours after the first injection granules can also be observed in the walls of the smaller omental blood vessels. They pass through the vascular walls and appear in the perivascular

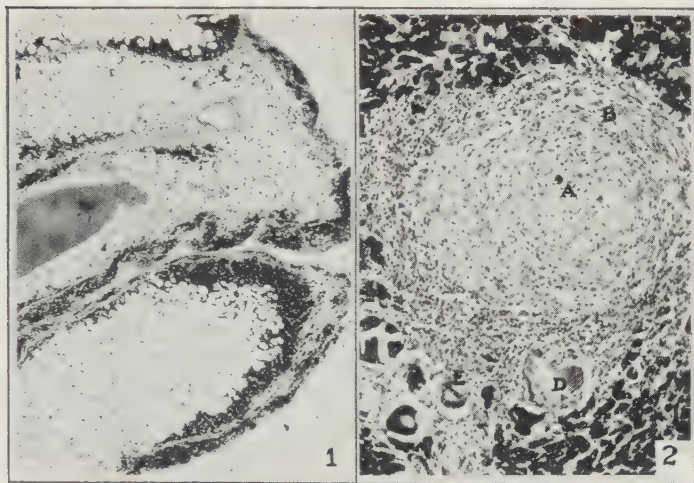


FIG. 1.

Omentum of an animal injected with India ink. Note folding of surface and sub-mesothelial macrophages.  $\times 25$ .

FIG. 2.

Tasche laiteuse showing: A. Central reticular zone; B. Lymphocytoid zone; and C. Phagocytic zone; D. and E. are foreign body giant cells.

loose connective tissue where they seem to excite the same histiocytic response as do the cells which enter by surface transmission, as evidenced by a cellular migration, transformation of the migratory cells into macrophages, and phagocytosis of the granules. The process leads eventually to formation of dense masses of loaded perivascular macrophages.

Definite reactive changes appear in the *tasches laiteuses*, particularly after a protracted series of pigment injections. The process culminates in the production of numerous macrophages, which emerge from the periphery of the milky spots. Under these conditions the activated spots consist of three concentric zones which merge into each other: (1) a light central zone made up of reticular cells; (2) a zone packed with cells of lymphocytoid type; (3) an outer zone in which these cells undergo gradual transformation into phagocytes which engulf the free pigment. There is a close correlation between the loss of lymphocytoid characteristics in the nucleus and the assumption of phagocytic power by these cells (Fig. 2).

Cytologic examinations of omenta removed while phagocytosis was taking place show a series of changes in the histiocyte which culminate in assumption of phagocytic power. Under favorable conditions all the changes can be observed in a single section, but



their interpretation and chronological arrangement are naturally subject to error on the part of the observer. This transformation seems to occur as follows:

(1) At the advent of mobilization the ordinary histiocyte increases in size and begins to show cytoplasmic vacuolation. Still more striking is the alteration in the configuration of the Golgi apparatus, first observed by Nassonov.<sup>3</sup> The reticular material of



FIG. 3.

Active macrophage showing a complex vacuole with pigment, and fragmented reticular material at its periphery. Ludford's fixation after trypan blue.

FIG. 4.

Macrophage with increased quantity of Golgi material, some of which is distributed about pigment vacuoles (A). B represents newly formed pigment clumps.

FIG. 5.

Macrophage showing numerous India ink granules. Pale rings about the larger masses represent Golgi material in negative. Bouin; Hematoxylin-eosin.

FIG. 6.

Macrophage showing incoming India ink particles (A), and condensing granules which will form a pigment mass. Note that some of the reticular material is enclosed in the mass, showing as pale centers. Bouin; hematoxylin-eosin.

<sup>3</sup> Nassonov, D., *Z. f. Zellforsch u. mikr. Anat.*, 1925, **3**, 472.



the resting histiocyte, which is in the form of a small, closely meshed net applied to the nuclear membrane, increases in size, becomes less closely meshed, and undergoes partial fragmentation. Isolated osmiophilic strands can soon be found distributed throughout the cytoplasm. By the time these changes have occurred, the cell is able to phagocytose particles, as is shown by the occasional presence of pigment granules in the cytoplasm (Fig. 3).

(2) Observations show that when the granule enters the cytoplasm it is soon enclosed in a fluid vacuole, which seems to attract the nearest Golgi fragments. Later the strands become so closely applied to the periphery of the vacuole as to form a continuous osmiophilic shell (Fig. 4).

(3) When two or more of the structures described, each of which is composed of a pigment granule in a fluid vacuole surrounded by an osmiophilic shell, come into contact with one another, they rapidly fuse. This process does not seem to take place prior to the formation of the osmiophilic shell (Figs. 5 and 6). When the contact is established, the pigment granules fuse and the shell disintegrates into discrete droplets deposited in the neighborhood of the developing pigment mass. The Golgi material seems to serve merely as an aid in the process of fusion; there is no indication that any part of it is incorporated in the fused mass.

A study of the many types of cells occasionally found to contain particles of the injected material showed that only during the process of active collection and storage of foreign pigment (true phagocytosis) was there any Golgi reaction to the presence of the ingested particles. The configuration of the reticular matter remained unchanged in the cells which merely transmitted the pigment. The reaction of the Golgi material can therefore be used as an index of phagocytosis.

*Summary and Conclusions.* The rat omentum, following the injection of particulate matter of various types, undergoes hypertrophy due to accumulation of histiocytes which are transformed into macrophages. The macrophages collect just beneath the surface and phagocytose the material which enters through and between the mesothelial cells. Secondary collections of macrophages appear about the smaller blood vessels and take up the particles which have traversed the vascular walls. The taches laiteuses increase in size and produce numerous macrophages.

With the advent of phagocytic activity the Golgi net increases in size and undergoes fragmentation. The fragments become asso-

ciated with the ingested granules and apparently aid in bringing about their coalescence into masses which are stored by the cells. The changes found in the Golgi material never occur in cells which do not collect and store particulate matter.

## 13159

**Excretion of Thiamin and its Degradation Products in Man.\***

HERBERT POLLACK, MAX ELLENBERG AND HENRY DOLGER.

*From the Medical Services and Department of Laboratories, Mount Sinai Hospital, New York City.*

The development of a simple procedure<sup>1</sup> to determine both thiamin and the pyrimidines in human urine permits the investigation of the possible relationship between the two.

Schultz, Atkins and Frey showed that the rate of glucose fermentation by a yeast is directly proportional to the concentration of thiamin present. In addition to thiamin, the pyrimidines give mol for mol stimulation effect on the rate of fermentation. Such pyrimidines are found normally in human urines. Since the pyrimidine nucleus constitutes an integral part of the thiamin molecule, it is of interest to determine the relationship between thiamin and urinary pyrimidine.

The total fermentation is a measure of thiamin and pyrimidine in the urine. The fermentation after oxidation of the free thiamin is a measure of the pyrimidines; the difference represents the free thiamin. This technic combines the gas method with the initial steps of the thiochrome procedure.

The urinary excretion of the thiamin and pyrimidine of a group of patients was studied under various conditions. Three patients were given a diet completely deficient in B<sub>1</sub> for 10 days. Two of these were normal and one had ileo-jejunitis. Fig. 1 and 2 and Table I illustrate that complete deprivation of dietary thiamin for a period of 10 days changed the thiamin-pyrimidine ratio from approximately 9:1 to 1:9. During this 10-day deprivation period,

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\* This work was supported in part by the Williams-Waterman Fund.

We are indebted to Dr. Charles Frey, of the Fleischman Laboratories, for his cooperation.

<sup>1</sup> Schultz, A. S., Atkin, L., and Frey, C. N., *Science*, 1938, **88**, 547; *J. Biol. Chem.*, 1940, **136**, 713.

TABLE I.  
Twenty-four-hour Excretion of Thiamin and Pyrimidine, During Administration  
of Low Thiamin Diet.  
S.F. Male. Age 13. Diagnosis: Ileo-colitis.

March	Total fermentation (mol-equivalents)	Vitamin B <sub>1</sub> (m-equiv.)	Pyrimidines (m-equiv.)	% of fermentation due to free thiamin
3	640	282	358	44
4	172	109	63	63
5	750	487	263	65
6	644	322	322	50
7	286	28	258	10
8	270	38	232	14
9	184	0	184	0
10	402	54	348	18

the absolute amount of pyrimidine excreted remained at approximately the same level, while the free thiamin disappeared almost completely. It becomes apparent, then, that the determination of free thiamin in the urine is an index of the dietary intake immediately preceding the measurement, since the urinary excretion of thiamin decreases so rapidly on deprivation. Therefore, low values for free thiamin need not necessarily indicate a state of chronic insufficiency with respect to this vitamin since the same values are obtained with temporary, acute deprivation. On the other hand, the combined gas-thiochrome method can distinguish the low values of thiamin resulting from acute or normal deprivation. If the thiamin excretion is low in the presence of normal pyrimidine excretion, it is indicative of recent deprivation. However, if both the thiamin and pyrimidine are below normal, it can be interpreted as indicating a protracted insufficiency of B<sub>1</sub>.

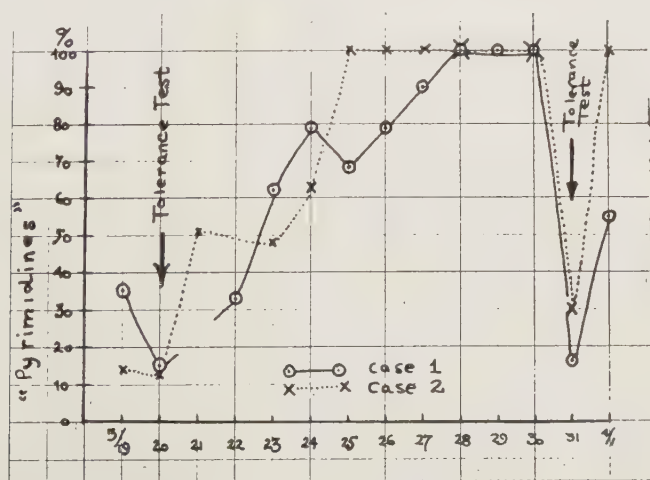


FIG. 1.

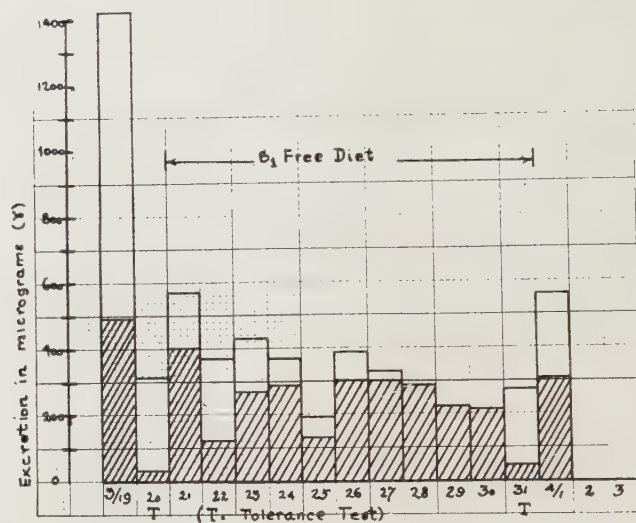


FIG. 2.

A further evidence in support of this concept is the result obtained with the 1 mg load test. By this test is meant the urinary response to the parenteral administration of 1 mg of thiamin hydrochloride, as previously reported.<sup>2</sup> This test was carried out before the experiment was started, and repeated after the 10-day deprivation period. It is highly significant that although the free thiamin was practically absent from the urine at the end of the 10-day period, the load test remained unchanged. This unchanged response to the 1 mg load test, (Fig. 2) indicates that the basic state of nutrition with respect to  $B_1$  was unaltered by the temporary deprivation in

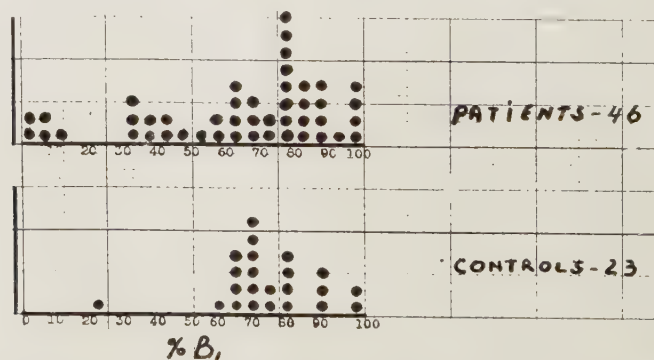


FIG. 3.

<sup>2</sup> Pollack, H., Dolger, H., Ellenberg, M., and Cohen, S., PROC. SOC. EXP. BIOL. AND MED., 1940, 44, 98.

a well-nourished individual. The laboratory data are supported clinically by the complete absence of any evidence of Vitamin B<sub>1</sub> deficiency either subjectively or objectively as measured by electrocardiogram, blood-sugar, cholesterol, total protein, hemoglobin, red blood cell count, blood pressure, pulse rate and weight.

Fig. 3 again demonstrates the importance of the pyrimidine concentration in relationship to the total thiamin excretion. When normal individuals are subjected to the 1 mg load test, over 60% of the yeast fermentation stimulating substance in the urine is free thiamin. Contrasting these results obtained from a group of unselected hospitalized patients, it is seen that 25% excrete more pyrimidine than thiamin. This indication that one-fourth of all patients admitted to the hospital showed atypical responses to the test is an independent correlation of a similar conclusion<sup>3</sup> arrived at by different means.

Table II shows the urinary excretion of pyrimidines following the daily parenteral administration of 100 mg of thiamin hydrochloride. It can be seen that the absolute amount of pyrimidine excreted is increased. This is evidence that urinary pyrimidine is derived from thiamin. Further, these data indicate that increasing the amount of thiamin administered increases its utilization, if the increased pyrimidine excretion in the urine is an index of actual utilization of thiamin. The administration of large doses of thiamin results in an absolute increase, but a relative decrease in the efficiency of utilization. Hence it might be suggested that frequent, divided dosage is the method of choice in administering this vitamin.

TABLE II.

Twenty-four-hour Excretion of Thiamin and Pyrimidine after Intravenous Administration of Vitamin B<sub>1</sub>.

T. McN. Male. Age 35. Diagnosis: Avitaminosis B-Complex.

Date	Total fermentation (mol-equivalents)	Vitamin B <sub>1</sub> (m-equiv.)	Pyrimidines (m-equiv.)	% of fermentation due to free thiamin
9/27	87,500	83,000	4,500	95
28	24,400	20,100	4,300	82
29	73,100	63,600	9,500	87
30	112,000	69,000	43,000	62
10/3	82,500	67,600	14,900	82
4	106,000	90,000	16,000	85
7	9,390	2,490	6,900	27
8	9,550	3,150	6,400	33
17	312,000	288,000	24,000	92
18	145,500	116,500	29,000	80
19	19,300	2,300	17,000	12

This patient received 100 mg thiamin daily intravenously from 9/27 to 10/16. On 10/16 he was given 300 mg, 10/17 200 mg, 10/18 no thiamin administered.

<sup>3</sup> Pollack, H., Ellenberg, M., and Dolger, H., *Arch. Int. Med.*, 1941, **67**, 793.



## Gastro-Intestinal Motility in the Albino Rat after Administration of Amphetamine Sulfate

ERMA SMITH\* AND K. E. PENROD

*From the Department of Zoology, Iowa State College.*

Reports concerning the action of amphetamine sulfate (A.S.) on gastro-intestinal motility are somewhat variable. Myerson and Ritvo<sup>1</sup> summarizing the results of their studies on man state that A.S. is a sympathomimetic drug which is of great value in diminishing or abolishing spasm of the G. I. tract. Ivy and Krasno,<sup>2</sup> summarizing the clinical literature state that 10-30 mg orally delays the rate of evacuation of the stomach, increases the tone of the pylorus, shows no marked constant effect on the small intestine and variable effects on the colon. Ersner,<sup>3</sup> after treating 500 cases of obesity with the drug, reports it to cause mild constipation, in 10 mg doses.

*Experimental.* Our study of A.S. consisted in measurement of the action of various doses on effective peristalsis and egestion time in intact animals and its action on isolated segments of colon.

The animals were fasted for 4 hours before each determination in order that they would quickly eat their test meal which consisted of a 3 g pellet made up of the following parts by wt: bread 1, milk 4, hamburger 5, and  $\text{Fe}_2\text{O}_3$  1. The A.S. dissolved in water was injected intraperitoneally, immediately after the food pellet was eaten.

The action on effective peristalsis<sup>4</sup> was measured by comparing the rate of progress of a test meal dyed with  $\text{Fe}_2\text{O}_3$  in treated and non-treated litter mates of the same sex, paired data being secured in all determinations; 30 min. after eating the animals were killed by decapitation, the gastro-intestinal tract quickly excised, straightened out on a warm moist plate and slit open with fine scissors; thus the distance which the dyed mass had been propelled from the stomach could be measured accurately.

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\* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

<sup>1</sup> Myerson, A., and Ritvo, Max, *J. A. M. A.*, 1936, **107**, 24.

<sup>2</sup> Ivy, A. C., and Krasno, I. R., *War Medicine*, 1941, **1**, 15.

<sup>3</sup> Ersner, Jack S., *Endocrinology*, 1940, **27**, 776.

<sup>4</sup> Patterson, C. A., Smith, Erma, and Hale, H. B., *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 509.

TABLE I.  
Effective Peristalsis.

No. of animals	Mg A.S. per kg/rat	Mean length of intestine traversed in % of total length	Range in %
45	0	70	64-96
20	5	45	0-51
20	10	33	0-46

The time required for first appearance of the  $\text{Fe}_2\text{O}_3$  in the egesta of animals fed the test meal with and without A.S. is summarized in Table II.

TABLE II.  
Egestion Time.

No. of determinations	Mg A.S./kg body wt	Egestion time in hr		% delay
		Mean	Range	
50	0	7	5-10	
10	4	11.5	10-15	64
10	5	10.5	10-16	50
30	10	12.9	10-16	84

From Tables I and II it may be seen that in all cases treatment with A. S. delayed passage of material along the gastro-intestinal tract in intact animals. Thirty min. after injecting the drug the stomach appeared atonic and the pylorus tightly constricted; casual measurement of the total length of the intestine from the pylorus to the anus in untreated rats showed the length to be about 70 cm; in treated animals it was 80 cm due to greater relaxation of the tube; 84% delay in egestion time was induced by administration of 10 mg per kg A.S.

In 100 tests using isolated segments of colon contracting in Sollman-Rademaeker's solution  $16\frac{2}{3}$  to  $33\frac{1}{3}$  gamma A.S. per cc added to the bath slowed or stopped the action of the strips. 35-45 gamma either stimulated or inhibited, whereas 50 gamma and above stimulated.

*Conclusions.* Amphetamine sulfate relaxes the stomach and small intestine, constricts the pylorus and delays the passage of material along the gastro-intestinal tract. Isolated segments of colon are stimulated by minimum effective doses; larger doses inhibit.

## Effect of Trypsin upon Blood Histamine of Rabbits.

CARL A. DRAGSTEDT AND M. ROCHA E SILVA.\*

*From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.*

It has been shown in previous experiments<sup>1</sup> that trypsin causes a contraction of various isolated smooth muscle structures such as the intestine of the guinea pig, rabbit, etc., and that this smooth muscle effect is characterized by a short latent period and by a subsequent desensitization, phenomena which are analogous to those seen when antigen is added to the bath containing the tissues of sensitized animals. To further amplify the analogy between the action of trypsin upon normal tissues and the action of antigen upon sensitized tissues, we have found that the addition of arginin or histidin to the bath will prevent or counteract the effects of trypsin just as it does the effects of antigen.<sup>2</sup> It has also been shown that the intravenous injection of trypsin into cats, dogs and rabbits produces a profound vascular shock similar in many respects to that of anaphylactic shock.<sup>3</sup> In addition, it has been shown<sup>4</sup> that the intravenous injection of trypsin into dogs leads to the liberation of histamine into the blood such as occurs in anaphylactic shock.<sup>5</sup>

Rose and Weil<sup>6</sup> found that the injection of antigen into sensitized rabbits leads to a reduction in the total blood histamine and this finding has been confirmed in our laboratory.<sup>7</sup> This apparently paradoxical behavior of the rabbit appeared to offer a suitable experiment by which the effects of trypsin could be compared further with those of antigen.

Crystalline trypsin,<sup>†</sup> in amounts varying from 3 to 7 mg per kilo, was injected via the marginal ear vein into 5 rabbits. Blood

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\* Guggenheim Fellow, Instituto Biológico, Sao Paulo, Brazil.

<sup>1</sup> Rocha e Silva, M., *Arch. f. exp. Path. u. Pharm.*, 1940, **194**, 335.

<sup>2</sup> Ackermann, D., *Naturwiss.*, 1939, 515.

<sup>3</sup> Rocha e Silva, M., *Arch. f. exp. Path. u. Pharm.*, 1940, **194**, 351.

<sup>4</sup> Ramirez de Arellano, M., Lawton, A. H., and Dragstedt, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 360.

<sup>5</sup> Dragstedt, C. A., and Gebauer-Fuelnegg, E., *Am. J. Physiol.*, 1932, **102**, 512, 520.

<sup>6</sup> Rose, B., and Weil, P., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 494.

<sup>7</sup> Dragstedt, C. A., Ramirez de Arellano, M., and Lawton, A. H., *Science*, 1940, **91**, 617.

<sup>†</sup> Plaut Research Laboratory preparation.

TABLE I.  
Histamine Content and Leukocyte Count of Rabbit's Blood Before and After the  
Intravenous Injection of Trypsin.

Exp.	Before		After	
	W.B.C.	Histamine	W.B.C.	Histamine
1	—	5.2	—	0.4
2	9,200	3.5	5,200	0.4
3	12,700	6.0	5,200	2.0
4	6,500	9.0	3,200	4.5
5	12,200	1.5	4,100	0.1

Histamine values in micrograms of histamine acid phosphate per cc of blood.

was obtained by cardiac puncture before and after the injection and assayed for its histamine content by Code's method.<sup>8</sup> Leukocyte counts were also made. The results are shown in the accompanying table.

As noted in the table, there was a striking reduction in the total blood histamine in every case. There was also an associated marked reduction in the leukocyte count. Code<sup>9</sup> has presented evidence that from 70 to 100% of the total blood histamine of rabbits is contained in the white blood cells and also<sup>10</sup> evidence that the granular leukocytes are the major source. From the results indicated in the table, it is apparent that the reduction in total blood histamine cannot be accounted for entirely by the reduction in the total leukocyte counts. As an alternative explanation for the much greater reduction in histamine than the corresponding reduction in leukocytes was obtained by studying the effects of trypsin on rabbit's blood *in vitro*, no attempts to correlate the histamine changes with counts of the granular cells were made.

Katz<sup>11</sup> demonstrated that when antigen is added to blood from a sensitized rabbit *in vitro*, there is a release of a considerable fraction of the cellular histamine into the plasma. We have found trypsin to have a similar effect. Rabbit's blood was obtained by cardiac puncture, heparinized to prevent clotting, and divided into 3 portions. The total blood histamine was determined on the first sample, the second sample was centrifuged and the plasma histamine determined, trypsin was added to the third sample and it was then centrifuged and the plasma assayed for histamine. The addition of trypsin caused no change in the total blood histamine, while the plasma

<sup>8</sup> Code, C. F., *J. Physiol.*, 1937, **89**, 257.

<sup>9</sup> Code, C. F., *J. Physiol.*, 1937, **90**, 349.

<sup>10</sup> Code, C. F., *J. Physiol.*, 1937, **90**, 485.

<sup>11</sup> Katz, G., *Science*, 1940, **91**, 221.

histamine was doubled or trebled, indicating that there must have been a release of histamine from cells to plasma. There is no reason to doubt that a similar release of histamine from cells to plasma occurs when trypsin is injected into the intact animal. In the latter instance the plasma histamine undoubtedly diffuses rapidly into the various tissues so that the reduction in total blood histamine which results when trypsin is injected into rabbits is partially due to the removal of leukocytes from the circulating blood and partially to the diffusion of histamine from blood cells to plasma to tissues.

*Summary.* The intravenous injection of trypsin into rabbits results in a prompt reduction in the total blood histamine with an associated leukopenia. The addition of trypsin to heparinized rabbit's blood *in vitro* results in a shift of histamine from cells to plasma. In both respects the effects of trypsin are similar to those produced by antigen in sensitized animals.

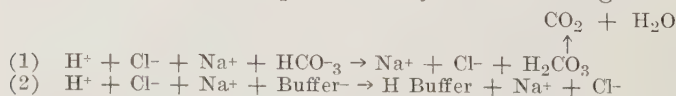
## 13162

### Relationship Between Osmotic Activity and Sodium Content of Gastric Juice.\*

NATHAN LIFSON, RICHARD L. VARCO AND M. B. VISSCHER.

*From the Department of Physiology, University of Minnesota.*

Several investigators<sup>1</sup> have advanced evidence for the view that gastric juice consists of a mixture of component secretions. Hollander has postulated at least two such components, one a solution of hydrochloric acid isosmotic with blood and the other an alkaline fluid with an electrolyte distribution more or less similar to that of plasma.<sup>2</sup> It was his conclusion that the mixture of these two component secretions would have a total ionic concentration less than either of the "pure" components. Under such circumstances buffering would occur as represented by the following reactions:



\*We are indebted to Mr. Charles Carr for carrying out the sodium determinations for this study.

<sup>1</sup> Gamble, James L., and McIver, Monroe A., *J. Exp. Med.*, 1928, **48**, 837; Hollander, Franklin, *J. Biol. Chem.*, 1932, **97**, 585; Dienst, C., *Ges. Exp. Med.*, **83**, 718; Liu, A. C., Yuan, I. C., and Lim, R. K. S., *Chinese J. Physiol.*, 1934, **8**, 1.

<sup>2</sup> Hollander, Franklin, *Am. J. Dig. Dis. and Nut.*, 1936, **3**, 651.



where  $\text{Na}^+$  represents a typical cation and "Buffer" represents any buffer anion other than bicarbonate. The resulting diminution of the concentration of osmotically active constituents should reflect itself in a decrease in osmotic activity of the mixture. In the present study, therefore, the osmotic activity of gastric juice samples has been plotted as a function of the sodium content, the latter being assumed tentatively to be a measure of the relative amounts of alkaline component in the mixed juice.

*Methods.* Medium size, healthy, mongrel dogs, possessing a functioning Heidenhain and/or pyloric pouch which had been constructed a minimum of 3 months prior to these experiments, were employed. All dogs were trained to remain standing for the duration of a particular experiment. Gastric secretion was stimulated by a subcutaneous injection of 1.5 mg of histamine diphosphate, or 12 mg of pilocarpine, in aqueous solution. Secretions were collected in clean test tubes, capped by a rubber diaphragm, to reduce evaporation and external contamination, with a single perforation allowing free flow from the pouch via a mushroom catheter. Plasma was obtained from femoral arterial blood samples drawn at the conclusion of each gastric juice collection period. All blood was secured and centrifuged under oil. Heparin was employed as the anticoagulant. Osmotic activities were determined with the Hill-Baldes<sup>3</sup> thermo-electric osmometer at 37.5°C (except for 3 initial samples determined at 25°C) and in a gas phase of 5%  $\text{CO}_2$  in oxygen. Results are expressed in terms of milli-equivalents of NaCl per kilo of water. Samples were analyzed for sodium by the micro zinc uranyl acetate method of Dean.<sup>4</sup>

*Results.* Fig. 1 presents in graphic form the results of the determinations. The feature to which we wish mainly to call attention is emphasized by the curve drawn as an approximate fit to the data. Regardless of the condition of stimulation or of the type of pouch from which samples were obtained, the osmotic activity of the juice appeared, under the circumstances of this investigation, to be a function of the sodium content. Specifically, (1) the samples with low sodium content (less than 20 m.E./L.) *i. e.*, those presumably representing acid juices with relatively little admixture of alkaline component, showed osmotic activities of the same order as, or higher than, that of the corresponding plasma; (2) the samples with the highest sodium contents (120-135 m.E./L.) *i. e.*, presumably those representing the alkaline component with relatively little admixture

<sup>3</sup> Baldes, E. J., *J. Sc. Instrum.*, 1934, **11**, 223.

<sup>4</sup> Dean, R. B., unpublished.

of acid juice, likewise tended to exhibit an osmotic activity of the same order as, or higher than, that of plasma; (3) samples with intermediate values were, with a few exceptions, hypotonic. It is precisely in these samples that a lowering of osmotic activity due to buffering would, by prediction, be most extensive. Therefore the general distribution of experimental values appears consistent with the two-component theory and the implied buffering mechanism.

Certain other features of these results with reference to the conditions of stimulation may also be briefly noted: (a) Histamine stimulated juices were all 2-6 m.E./K. hypertonic (relative to the corresponding plasma); (b) pilocarpine stimulated juices were either

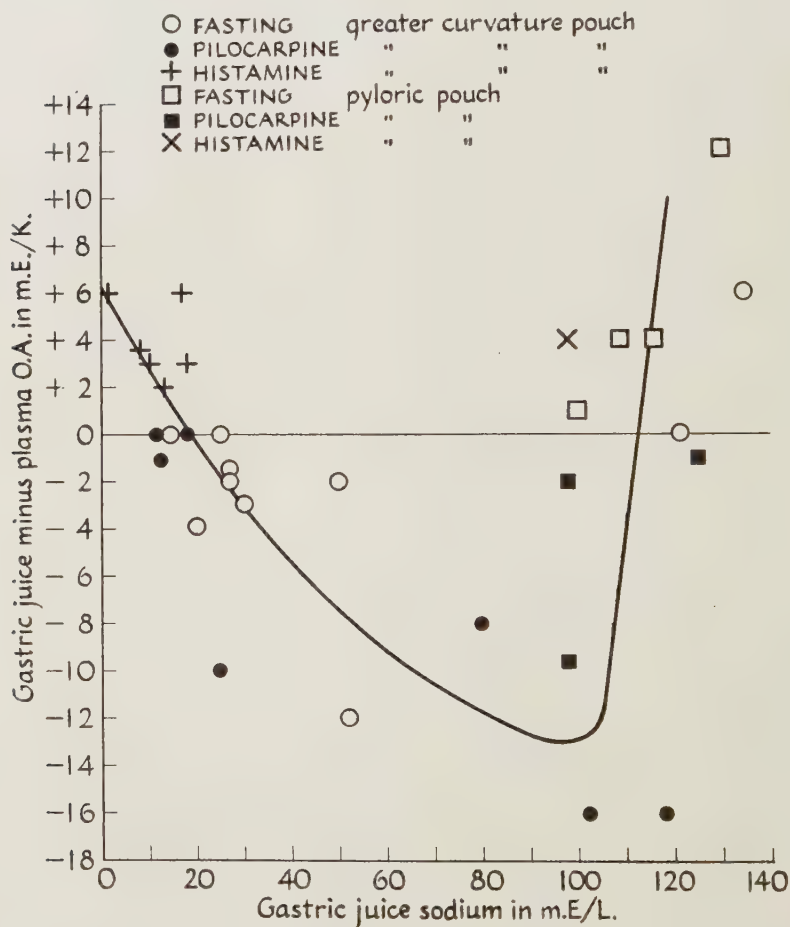


FIG. 1.

Relationship between relative osmotic activity of gastric juice and its sodium content.

isotonic of hypotonic, but not hypertonic; (c) fasting juices were hypertonic, isotonic, or hypotonic.

The mean osmotic activity of 30 plasma samples was 159.8 m.E./K. with a standard deviation of the distribution of  $\pm 2.8$  m.E. K.

*Summary.* The osmotic activity of pouch gastric juice samples relative to that of the plasma is presented as a function of the sodium content of the gastric juice.

The general features of the resulting distribution are in qualitative agreement with the predictions based on the two-component theory of Hollander and the buffering process which it implies.

### 13163

#### The First Appearance of Functional Activity in the Pars Intermedia in the Frog, *Xenopus*.

WILLIAM ETKIN.

*From the American Museum of Natural History and the College of the City of New York.\**

The development of functional activity in the pars intermedia of the pituitary has evoked considerable interest since the first workers on the amphibian gland found evidence of early activity in the gland. From the original observations of Smith and Allen (see Smith<sup>1</sup>) on hypophysectomy in the frog embryo it was apparent that the operated animals could be distinguished from the controls in pigmentation by the time the animal assumed its definitive tadpole form. In my own experience with *Rana sylvatica* and *pipiens* this is at about 13 mm and about 7-8 days after the tail bud stage at 26°C. Blount<sup>2</sup> in his multiple pituitary transplants in *Amblystoma* noted some differences in general tone of pigmentation between hypophysectomized, normal and multiple pituitary animals at earlier stages than reported in the frog. He interpreted his observations to indicate hypophyseal function at about stage 41 of Harrison, apparently the stage at which the limb buds

\* Assistance in the conduct of this work was provided by Works Progress Administration, Official Project No. 165-1-97-8WP10.

<sup>1</sup> Smith, P. E., *Amer. Anat. Mem.*, No. 11, 1920.

<sup>2</sup> Blount, R. F., *J. Exp. Zool.*, 1932, **63**, 113.

begin to show division. In the chick, Chen, Oldham and Geiling<sup>3</sup> recently reported extracting a potent melanophore factor from the pituitary regions of chicks of 5 days' incubation. This is a stage before cytoplasmic differentiation appears in the gland cells.

Through the courtesy of Mr. L. R. Aronson of this laboratory, the author has had the opportunity of observing the development of the tadpoles of *Xenopus laevis*, the so-called African clawed frog. It was noted that the egg has very little free pigment. Whether because of this or of a genuine precocity in appearance, the true melanophores can be observed at a relatively early stage of development. It was felt that the investigation of the initiation of secretion in the pars intermedia could be made at an earlier stage in embryogeny in this form than in any others hitherto reported.

Bles<sup>4</sup> has described the normal development of this animal and although he did not describe the history of the melanophores, his excellent figures of the stages furnish convenient reference points. Hypophysectomy was done on animals in early tail bud stages (Bles Fig. 12, 13) about 24 hours after egg-laying. No melanophores are discernible at this stage nor has heart beat appeared. Twenty operated and a like number of normal animals were set up.

Since it was not possible to observe these animals continuously, accurate timing of development at a constant temperature was not made but in approximately 20 hours at room temperature the animals passed into advanced tail bud stages as shown in Bles Fig. 15a. In this stage the first appearance of melanophores can be seen along the anterior dorsal margin of the yolk mass. In both the normal and hypophysectomized animals these were observed to be in approximately the same state, namely, well expanded but perhaps not completely so. In the course of the next 5 or 6 hours the animals develop more elongate tails and reach the stage illustrated in Bles Fig. 19. This is slightly earlier than shown in Fig. 1 of the present paper. At this stage the normals show well expanded melanophores extending along the dorsal edge of the yolk body, on either side of the nerve cord and over the dorsal aspect of the anterior end of the brain. The successfully hypophysectomized animals, at this time, can be clearly distinguished from the normals by the fact that most of the melanophores are rather strongly retracted though not reduced to points. This is most clearly seen in the dorsal melanophores and is illustrated by an hypophysectomized animal in a slightly later stage

<sup>3</sup> Chen, G., Oldham, F. K., and Geiling, E. M. K., *Proc. Soc. Exp. Biol. and Med.*, 1941, **45**, 810.

<sup>4</sup> Bles, Ed. J., *Trans. Roy. Soc. Edin.*, 1906, **41**, 789.

in Fig. 1 and in Fig. 2 for a still older normal animal. The melanophores along the anterior margin of the yolk which had been the first to develop remain in a partially expanded condition for as much as a day after the dorsal melanophores show clear contraction. The development of 15 hypophysectomized and 15 control animals was followed through this stage.

In the subsequent development of the two groups the distinction between them becomes more and more marked so that when the definitive tadpole form is attained the well known characteristics of the hypophysectomized animal appear in their typical form; the melanophores are contracted and the leucophores are broadly expanded.

It is thus apparent that in late tail bud stages, 26 hours or so after the appearance of the pituitary primordium and about 48 hours after fertilization, the effect of a removal of that primordium can be clearly detected in the diagnostic reaction of the melanophores. The animal at this stage shows no limb buds, and only slightest stubs of gills. The oral membrane is not yet ruptured and circulation has

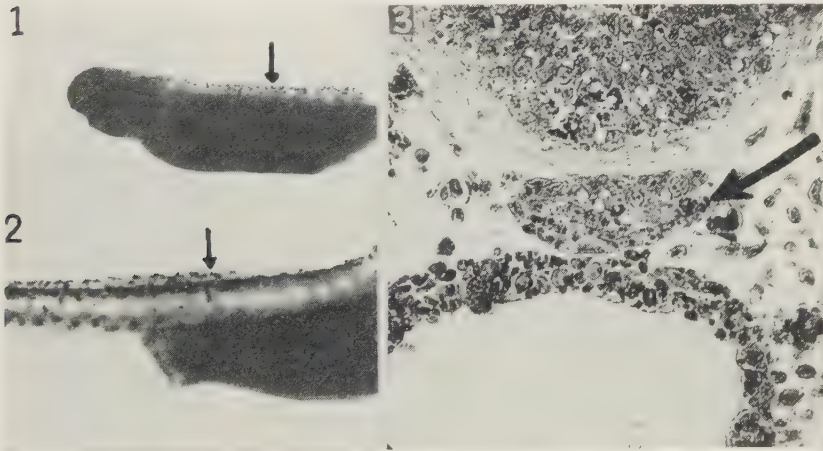


FIG. 1.

Hypophysectomized animal slightly beyond stage corresponding to Bles. Fig. 19. Note contracted dorsal melanophores such as near tip of arrow.

FIG. 2.

Normal animal a few hours older than in Fig. 1. Note expanded dorsal melanophores as near tip of arrow. Contiguous melanophores appear to fuse to form a continuous band.

FIG. 3.

Section through pituitary primordium in animal same stage as in Fig. 1. Pituitary may be seen between foregut below and diencephalon above. Yolk in pituitary appears as irregular granules larger than the chromatin granules. One group of such is to be seen just beyond tip of the arrow.



been established less than 18 hours. In accordance with our present concepts this may be taken to indicate that at this time, and possibly even earlier, the melanophore hormone is already being produced and secreted by the developing gland. The morphology of the gland at this period is therefore of some interest. In sections it appears as a mass not differentiated into parts and still showing remnants of a column of cells connecting to the ectoderm. The cells still contain considerable yolk granules, the cytoplasm is scant and does not show granular differentiation (Fig. 3). We are thus led to conclude that melanophore hormone secretion is initiated in the still undifferentiated primordium of the pituitary in *Xenopus*.

*Summary.* Observations on the development of the melanophores in normal and hypophysectomized *Xenopus* show contraction of the melanophores in the latter at a late tail bud stage, about 48 hours after fertilization. The pituitary at this stage is undifferentiated. It is inferred that the secretion of melanophore hormone begins at or possibly before this time.

## 13164

### Selective Inhibition of Sulfonamide Drugs by Various Media.

ELIAS STRAUSS AND MAXWELL FINLAND.

*From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston.*

It is now recognized that body fluids, as well as the usual laboratory media for cultivation of bacteria, contain substances which inhibit the action of sulfonamide drugs.<sup>1, 2</sup> It has been shown that different media vary in the amount of inhibiting substances they contain.<sup>2</sup> The question arises whether different sulfonamide drugs are inhibited to the same degree in a given medium. Were this true it might be possible to draw valid conclusions from *in vitro* studies of the comparative bactericidal or bacteriostatic effects of different drugs. In this paper we present some evidence that this assumption may not be justified.

*Methods and Materials.* Growth curves with stock strains of

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<sup>1</sup> Lockwood, J. S., and Lynch, H. M., *J. Am. Med. Assn.*, 1940, **114**, 935.

<sup>2</sup> MacLeod, C. M., *J. Exp. Med.*, 1940, **72**, 217.

Type III and Type V pneumococci were made as in previous studies.<sup>3</sup> In making explants for colony counts, the bacteriostatic effect of sulfonamide drugs was inhibited by the addition of *p*-aminobenzoic acid.<sup>4</sup> The "blood broth" consisted of a meat infusion broth with 1% bacto-peptone (Difco) and 0.05% glucose, to which 1% defibrinated rabbit blood was added. "Liver infusion" was prepared as described by MacLeod,<sup>2</sup> but modified by the addition of 1% of an acid hydrolysate of casein\* and suitable buffering salts.

**Results.** The comparative effects of equal concentrations of sulfathiazole and sulfadiazine on the growth of Type III pneumococci in various media are shown graphically in Fig. 1.

In blood broth, with inocula of about 100 organisms per milliliter, 10 mg % of sulfathiazole are bactericidal in 24 hours. Under identical conditions, sulfadiazine is only slightly bacteriostatic. In horse serum the results are about the same as in blood broth. In human plasma (cell-free citrated blood) sulfadiazine exhibits relatively more bacteriostasis, and sulfathiazole relatively less, than in blood

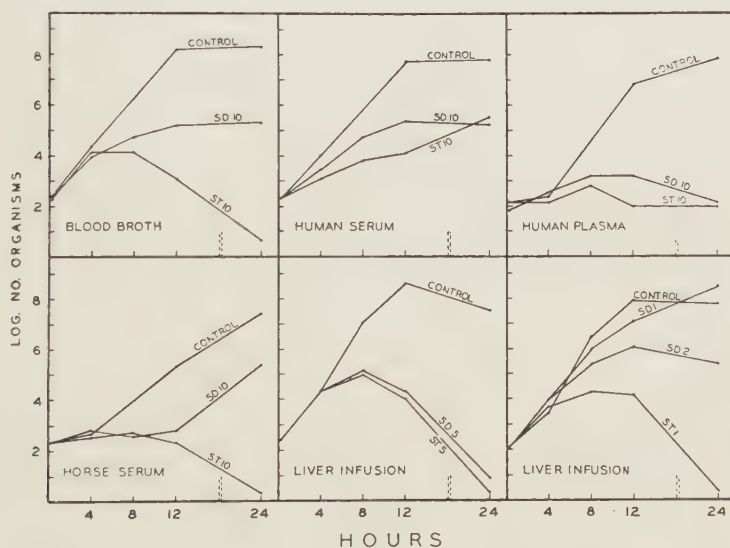


FIG. 1.  
Growth curves of Type III pneumococcus in various media. S.D. = sulfadiazine, S.T. = sulfathiazole, and the numbers indicate the concentration of these drugs in mg per 100 ml. Control contains no drug.

<sup>3</sup> Spring, W. C., Jr., Lowell, F. C., and Finland, M., *J. Clin. Invest.*, 1940, **19**, 163.

<sup>4</sup> Strauss, E., Lowell, F. C., and Finland, M., *J. Clin. Invest.*, 1940, **20**, 189.

\* Prepared and supplied by Dr. J. Howard Mueller.

broth. On the other hand, in human serum (from clotted blood) sulfathiazole is inhibited to a much greater degree than in any of the other media, while sulfadiazine is about as effective as it is in blood broth or in horse serum. In the liver infusion medium the two drugs are equally bactericidal in concentrations of 5 mg % or more. In this medium, however, sulfathiazole is bactericidal in concentrations as low as 1 mg %, whereas a similar concentration of sulfadiazine is almost without effect.

In Fig. 2 a comparison is made of the effect of sulfathiazole and sulfadiazine on the growth of Type V pneumococci in whole defibrinated blood and in the serum of the same defibrinated blood. In previous tests this subject's blood was found to possess no natural bactericidal action against Type V pneumococci. Here again sulfathiazole was more effective in defibrinated blood than in serum. Sulfadiazine, on the other hand, was only slightly bacteriostatic in both media.

*Comment.* Various types of *in vitro* tests have been used for the determination of the comparative bactericidal and bacteriostatic efficacy of sulfonamide drugs.<sup>5-8</sup> In studies on the action of sulfapyridine and sulfathiazole on the pneumococcus, both the clinical results and therapeutic experiments in animals correlated fairly closely with *in vitro* results. With sulfadiazine it became evident

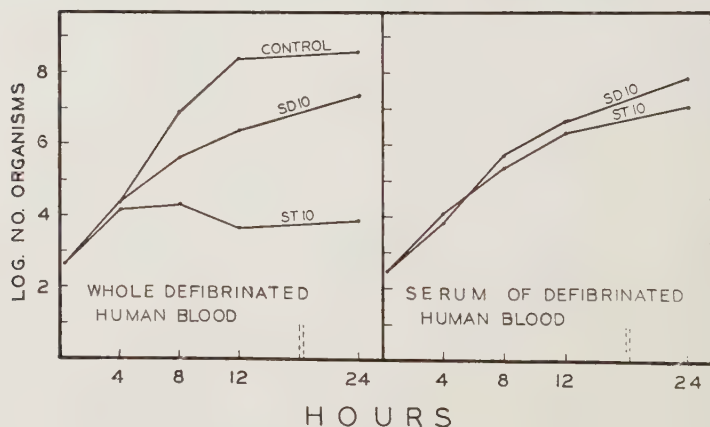


FIG. 2.

Growth curves of Type V pneumococcus in defibrinated blood and in its serum. S.D. = sulfadiazine, S.T. = sulfathiazole, and the numbers represent concentration of the drugs in mg per 100 ml. Control contained no drug.

<sup>5</sup> Chu, H. I., and Hastings, A. B., *J. Pharmacol. and Exp. Therap.*, 1938, **63**, 407.

<sup>6</sup> Kempner, W., Wise, B., and Schlayer, C., *Am. J. Med. Sci.*, 1940, **200**, 484.

<sup>7</sup> Long, P. H., and Bliss, E. A., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 324.

<sup>8</sup> Lowell, F. C., Strauss, E., and Finland, M., *Ann. Int. Med.*, 1940, **14**, 1001.

that the excellent therapeutic results in animals<sup>9</sup> and in patients<sup>10, 11</sup> were at variance with the results obtained *in vitro* with the media usually employed for these tests. The results reported in this paper emphasize the importance of the proper selection of media for the performance of *in vitro* tests. Both sulfathiazole and sulfadiazine are inhibited to various degrees in the media used; moreover, the degree of inhibition of the same drug is not constant in different media. On the basis of activity in blood broth it might be concluded that sulfadiazine is no more active against Type III pneumococci than an equivalent concentration of sulfanilamide. Essentially the same results were obtained by Osgood, using marrow cultures.<sup>12</sup> In a liver infusion medium, however, sulfadiazine is as active as sulfathiazole in concentrations of 5 mg % or more. In lower concentrations in this medium our unpublished observations indicate that it is about as active as sulfapyridine.

*Summary.* Sulfathiazole and sulfadiazine are inhibited to varying degrees in different media. In blood broth sulfadiazine is selectively inhibited to a greater degree than sulfathiazole. In human serum sulfathiazole is inhibited to a greater degree than it is in blood broth, in human plasma, in defibrinated blood or in horse serum, while sulfadiazine is inhibited to about the same extent in these media. In liver infusion medium sulfadiazine and sulfathiazole are equally effective in a concentration of 5 mg % but sulfathiazole is more effective in lower concentrations. The possible error of attempting to compare therapeutic efficacy of different sulfonamides on the basis of the results of *in vitro* tests is discussed and the importance of the proper selection of media for the performance of *in vitro* tests of sulfonamide drugs is emphasized.

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<sup>9</sup> Feinstone, W. H., Williams, R. D., Wolff, R. T., Huntington, E., and Crossley, M. L., *Bull. Johns Hopkins Hosp.*, 1940, **67**, 427.

<sup>10</sup> Flippin, H. F., Rose, S. B., Schwartz, L., and Domm, A. H., *Am. J. Med. Sci.*, 1941, **201**, 585.

<sup>11</sup> Finland, M., Strauss, E., and Peterson, O. L., *J. Am. Med. Assn.*, 1941, **116**, 2641.

<sup>12</sup> Bullowa, J. G. M., personal communication.

## Bacteriostatic and Bactericidal Action of Sulfadiazine *in Vitro* on Gram-Negative Bacteria.

ELIAS STRAUSS AND MAXWELL FINLAND.

*From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston.*

Animal studies<sup>1</sup> and clinical results in patients<sup>2,3</sup> have indicated that sulfadiazine may be a chemotherapeutic agent of low toxicity and wide therapeutic applicability. *In vitro* studies of this drug on the pneumococcus are reported separately.<sup>4</sup> In this paper a comparison is made of the bactericidal and bacteriostatic activity *in vitro* of sulfadiazine and sulfathiazole on a number of common pathogenic gram-negative bacteria in a simple medium.

**Materials and Methods.** Stock laboratory strains of *Escherichia coli*, *Salmonella typhimurium* (aertrycke), *S. enteritides*, *S. paratyphi*, *S. schottmülleri*, *S. choleraesuis* (suipestifer), *Klebsiella pneumoniae* (type A) and *Shigella paradysenteriae* (Flexner) were used. The identity of the organisms was verified by their typical cultural, fermentation and serological reactions.\* The medium used was that of Sahyun as modified by MacLeod<sup>5</sup> by the addition of 0.2% of an acid hydrolysate of casein. Growth curves were performed by the method previously described.<sup>6</sup> Incubation was at 37°C. The stock cultures were stored at 4°C. on plain agar slants and were transferred 2 or 3 times in the semi-synthetic medium before testing.

**Results.** The comparative activity of sulfadiazine and sulfathiazole on the organisms tested is shown in Fig. 1. With original inocula of from 100 to 1000 organisms per milliliter, 1 or 2 mg

<sup>1</sup> Feinstone, W. H., Williams, R. D., Wolff, R. T., Huntington, E., and Crossley, M. L., *Bull. Johns Hopkins Hosp.*, 1940, **67**, 427.

<sup>2</sup> Finland, M., Strauss, E., and Peterson, O. L., *J. Am. Med. Assn.*, 1941, **116**, 2641.

<sup>3</sup> Flippin, H. F., Rose, S. B., Schwartz, L., and Domm, A. H., *Am. J. Med. Sci.*, 1941, **201**, 585.

<sup>4</sup> Strauss, E., and Finland, M., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 428.

\* The strains were obtained from Miss Marian Lamb of the Bacteriological Laboratory, Boston City Hospital. The medium used was unsatisfactory for studies on the stock strain of *Eberthella typhi*.

<sup>5</sup> MacLeod, C. M., *J. Exp. Med.*, 1940, **72**, 217.

<sup>6</sup> Spring, W. C., Jr., Lowell, F. C., and Finland, M., *J. Clin. Invest.*, 1940, **19**, 163.



% of either drug is bactericidal in 24 hours against *E. coli*, *Shigella paradysenteriae* (Flexner) and *S. choleraesuis* (suipestifer). For *S. enteritidis*, *S. schottmülleri* and *S. typhimurium* (aertrycke), 5 mg % is bactericidal; for *S. paratyphi* this concentration is bacteriostatic. In the case of *Klebsiella pneumoniae* (type A) both drugs are bactericidal in concentrations of 10 mg %.

*Comment.* The marked bacteriostatic and bactericidal activity of both drugs in concentrations as low as 1 mg % is in contrast to their only moderate bacteriostatic effect in broth in concentrations from 10 to 50 times as great.<sup>7</sup> In studies to be reported separately, we have tested various sulfonamide drugs on *E. coli* in a completely synthetic medium in which inorganic salts are the only source of nitrogen. The activity of sulfadiazine and sulfathiazole is essentially as reported here, while sulfapyridine, sulfaguanidine and sulfanilamide are progressively less active in the order given. In the case of *E. coli*, therefore, there is no evidence that there is any significant degree of inhibition of sulfadiazine and sulfathiazole by the medium used in these studies. The two drugs probably have about the same order of activity for this organism. Whether or not the medium used here had any inhibitory effect on the drugs in the case of the other organisms studied cannot be stated.

Some of the difficulties involved in attempting to predict thera-

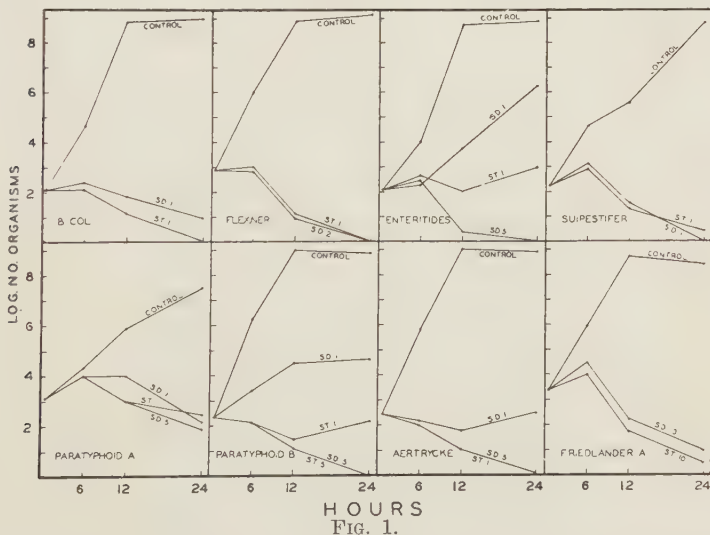


FIG. 1.

Growth curves of 8 strains of gram-negative bacteria in a simple medium. Control contains no drug. SD = sulfadiazine, ST = sulfathiazole, and the numbers indicate the concentration of these drugs in mg per 100 ml.

<sup>7</sup> Klinefelter, H. F., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 591.

peutic efficacy on the basis of *in vitro* results of sulfonamide action have been commented upon elsewhere.<sup>4</sup> The activity of a drug *in vitro* does not necessarily parallel its activity in experimental animals or in human subjects. *In vitro* tests may be useful as preliminary guides to *in vivo* studies, but cannot substitute for them.

*Summary.* When small inocula are planted in a semi-synthetic medium, sulfadiazine and sulfathiazole are bactericidal in concentrations as low as 1 or 2 mg % on *E. coli*, *Shigella paradyenteriae* (Flexner) and *S. choleraesuis* (*suipestifer*); and in 5 mg % on *S. enteritides*, *S. schottmülleri* and *S. typhimurium* (*aertrycke*). In the latter concentration they are bacteriostatic for *S. paratyphi* and in 10 mg % they are bactericidal for *Klebsiella pneumoniae*, type A.

## 13166

### Radiosensitivity of Skin of New-Born Rats. III. Sensitivity at Different Temperatures.\*

T. C. EVANS, J. P. GOODRICH, AND J. C. SLAUGHTER.

(Introduced by J. H. Bodine.)

*From the Departments of Radiology and Zoology, State University of Iowa.*

In a previous paper<sup>1</sup> it has been reported that temperatures of 0-5°C during roentgen irradiation (1,300 r) decreased the amount of injury produced in the skin of new-born rats. In a second series of experiments<sup>2</sup> this increased resistance of animals irradiated at 0-5°C was studied quantitatively for dosages ranging from 300 to 3000 roentgens. The purpose of this investigation was to determine whether the resistance would change quantitatively with increasing temperature.

The animals were of the same strain used in the previous studies.<sup>†</sup> The temperature was controlled during the irradiation by placing the animals in a double walled box, constructed of transparent sheet plastic, containing water at the desired temperature. The

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\* Aided by grants from the Committee on Radiations of the National Research Council, and the Rockefeller Fund for Research on Physiology of the Cell.

The writers are indebted to Professors J. H. Bodine and H. D. Kerr for their interest and helpful criticisms.

<sup>1</sup> Evans, T. C., *J. Roentgenol. and Rad. Ther.*, in press.

<sup>2</sup> Evans, T. C., Robbie, W. A., Goodrich, J. P., and Slaughter, J. C., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 662.

<sup>†</sup> These animals were kindly supplied by Dr. Emil Witschi from his rat colony.

temperature of the skin before and after the irradiation was determined by means of a thermocouple. Experiments indicated that the rectal temperature changed very rapidly to that of the skin at low and at high temperatures, but at room temperature the interior of the body was slightly warmer than the skin.

The radiation used was unfiltered except for 1 mm of cardboard and 2 sheets of 0.5 mm plastic. The radiation was delivered at 130 K.V. peak and 5 ma. The half-value layer was ca. 1.5 mm aluminum. The intensity in the box at 26°C was 137 roentgens per minute. The target distance was 30 cm.

The exposed skin was a 6 x 9 mm area to one side of the mid-ventral region of the abdomen. Both ventral and dorsal skins were examined microscopically 2 weeks later, and the extent of injury determined in units as described in a previous report.<sup>2</sup>

Fig. 1 shows some typical results of irradiation at different temperatures. These animals were litter mates and each was given the same dosage. No epilation is visible in the animals treated at 0° and 6°C. The rat irradiated at 17°C shows slight epilation and the one at 37°C shows complete epilation. Twenty-five new-born rats have been irradiated in this investigation, and the results are summarized in Fig. 2. The width of each block indicates the variation in temperature from the beginning to the end of the irradiation and the height of each block is twice the standard error of the mean of the determinations as made several times by each investi-



FIG. 1.

Littermates 14 days after irradiation with a dosage of 2010 roentgens.

gator. The highest temperature employed was 40°C which was as high as the animal could tolerate for the 15-minute period. Although much individual variation is evident, the data clearly show that the sensitivity increases as the temperature is raised. It is interesting to note that the variation produced at the different temperatures covers a range of injury which is equivalent to that produced by 0 to 2500 roentgens.

The quantitative relationship between the effect and the temperature indicates the possible importance of one or more metabolic processes in determining the amount of injury produced by the radiation. Although the heart rate, breathing, etc. of such new-born

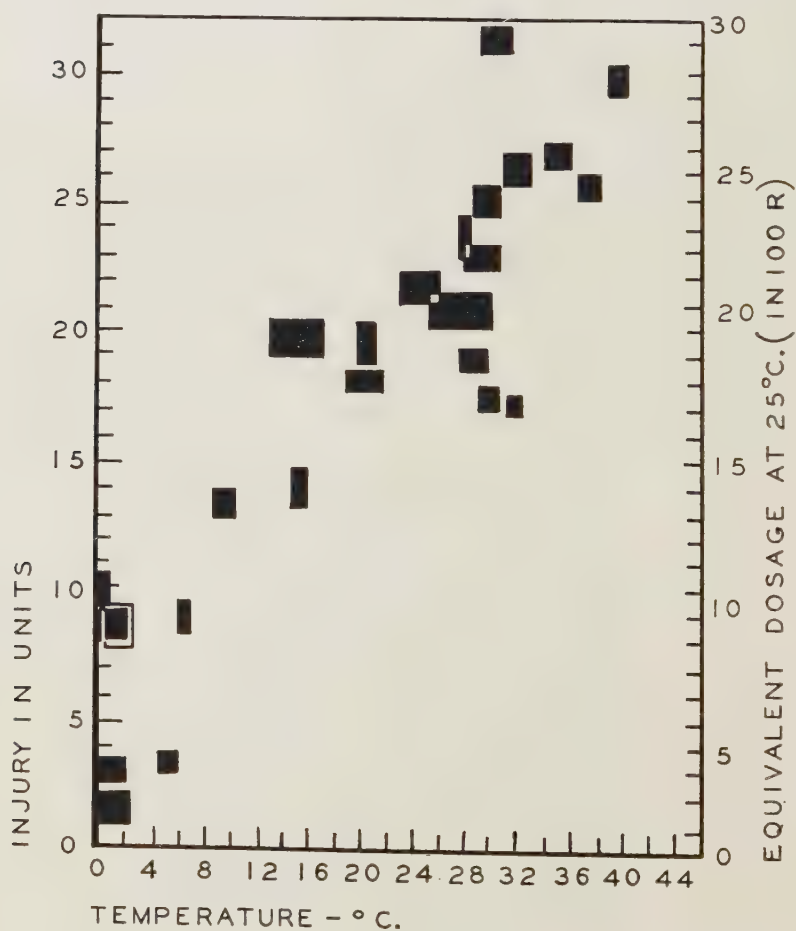


FIG. 2.

A graph showing the amount of injury produced at different temperatures.

animals are generally regarded as dependent on temperature, it seemed worthwhile to test the relationship on these animals.

With the assistance of Professor T. L. Jahn, electrocardiograms were made at different temperatures. The first experiment was begun at 26.5°C and the heart was beating 225 times a minute at this temperature. The temperature was then lowered to 16.5° and the heart beat rate immediately dropped to about 125 per minute. After the animal had been at 10° for 10 minutes the rate was about 30 per minute. The rate after 30 minutes at 10° was only 18 per minute. The temperature was then reduced to 1-2°C and the rate dropped to 2 per minute. The temperature was raised to 26.5° and after an hour the rate had increased to 250 per minute. The rate at 35° was 350 and at 40° the maximum of 400 beats per minute was obtained. A temperature of 45° proved to be too warm, as the rate dropped from 375 to 100 in 15 minutes, and after 5 more minutes the rat was dead.

In another experiment, a new-born rat was placed on ice and the heart-beat rate was found to reach the minimum within 10 minutes. This corresponds roughly to the rate of cooling of the skin. After it had been held to the minimal rate for 20 minutes the animal was placed again at room temperature. Within 5 minutes the rate had increased from zero to 22 per minute. After 53 minutes at room temperature the rate had increased to 165 beats per minute.

The rate of breathing and the rate of oxygen consumption were also found to be increased by raising, and decreased by lowering the temperature.

Since the circulation and respiration were found to respond to temperature in much the same way as the radiosensitivity it is quite possible that the temperature exerts its influence on radiosensitivity through its control of metabolic processes such as these.



## Vitamin K Storage and Prothrombin Levels in Chicks Obtained from Injected Eggs.\*

R. T. TIDRICK, F. W. STAMLER, F. T. JOYCE, AND E. D. WARNER.

*From the Department of Pathology, State University of Iowa, Iowa City.*

It has been shown<sup>1</sup> that hatching eggs obtained from hens maintained on a diet rich in vitamin K yield chicks which appear to contain stores of the vitamin. Such chicks, when placed on a vitamin K-free diet show evidence of some protection against the development of "hemorrhagic chick disease." Also, seasonal variations in the severity of this disorder have been noted.<sup>2</sup> Such variation might well be due to seasonal variation in the amount of vitamin K in the diet of the laying hens.

In an attempt to study storage of vitamin K in greater detail, we have conducted a number of experiments in which vitamin K was injected into the eggs prior to their incubation. Certain of the synthetic compounds, now readily available, can be injected into the eggs without seriously interfering with the hatching yield or with the vigor of the newly-hatched chicks. In the present paper we wish to present data obtained by injecting potassium 2-methyl-1,4-naphthohydroquinone disulfate into the eggs.

The vitamin was dissolved in phosphate buffer (pH 7.05) and heated to the boiling point briefly to reduce the likelihood of bacterial contamination. In each instance, the dose injected was contained in 0.2 cc of the buffer solution. The eggs, immediately prior to their incubation, were cleansed with alcohol and the vitamin was injected into the albumin portion. The opening in the shell, made with a diamond point pencil, was sealed with celloidin. The two vitamin K levels used in the present experiment were 200  $\mu$ g and 1000  $\mu$ g. The hatch yielded 15 chicks from eggs injected with 1000  $\mu$ g, 10 chicks from the eggs injected with 200  $\mu$ g, and 18 uninjected controls. At the time of hatching, all of the chicks were

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\* Aided by a grant from the John and Mary R. Markle Foundation and by funds provided by the Graduate College of the State University of Iowa.

The vitamin K preparation used in this study was kindly prepared in the Organic Chemistry Department of the University under the direction of Dr. G. H. Coleman.

<sup>1</sup> Almquist, H. J., and Stokstad, E. L. R., *J. Nutrition*, 1936, **12**, 329.

<sup>2</sup> Thayer, S. A., McKee, R. W., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 194.

placed on a vitamin K-free diet, previously described.<sup>3</sup> The blood samples were taken from the jugular vein with a syringe and hypodermic needle. Individual chicks were bled at 3-6-day intervals, so spaced as to give maximal coverage of the entire experimental period and at the same time avoid severe anemia. The prothrombin determinations were made by the two-stage method of Warner, Brinkhous and Smith.<sup>4-6</sup> A total of 152 determinations was made during this experiment.

The results obtained are shown graphically in Fig. 1. For simplicity in charting, the prothrombin values obtained were grouped into 5-day periods. The average prothrombin level and the average age of the chicks at the time tested were computed for each 5-day interval.

At the time of hatching, the prothrombin level of the control chicks is only slightly lower than that of the chicks obtained from the injected eggs. From statistical analysis and from unpublished data, we doubt that this difference is significant.

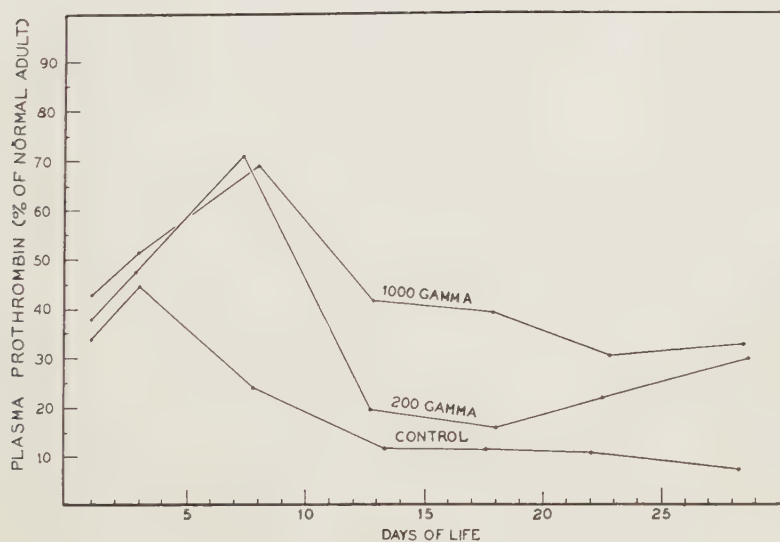


FIG. 1.

Prothrombin levels in chicks from injected eggs and from uninjected control eggs.

<sup>3</sup> Tidrick, R. T., Joyce, F. T., and Smith, H. P., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **42**, 853.

<sup>4</sup> Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

<sup>5</sup> Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, **66**, 801.

<sup>6</sup> Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1939, **125**, 296.

There is a small initial rise in the prothrombin level of the control group. This suggests the existence of small amounts of stored vitamin. This reserve is soon exhausted, however, and the prothrombin level falls markedly within the next 10 days. The chicks from the injected eggs, in contrast, contain sufficient stores that for a time the prothrombin level continues to rise toward adult levels which, by the two-stage prothrombin method, are somewhat greater than twice the hatching level. After about 10 days, this reserve is no longer adequate to maintain the rise but does prevent the prothrombin from rapidly falling to the bleeding level.

Statistical analysis of the results showed remarkably little individual variation in the prothrombin values of the control group. In the injected groups, there was considerable variation from chick to chick. Also, there was considerable overlapping between the two injected groups during the first 10 days of life. The prothrombin values of the injected chicks were, however, consistently higher than those of the controls, and after the first 10 days of life, there was a conspicuous difference between the two injection levels. For the 10-15-day period, the observed difference in the average prothrombin values between the 200  $\mu$ g and the 1000  $\mu$ g groups is approximately  $3\frac{1}{3}$  times the calculated standard error of the difference. During the next 5-day period, the observed difference between the average prothrombin values of these two groups is approximately 6 times the standard error of the difference. During these two age periods, the observed difference between the control group and the 200  $\mu$ g group is slightly greater than twice the standard error of the difference. Also, it is significant that during the entire 30-day period, none of the chicks from the injected eggs showed any evidence of spontaneous hemorrhage. In sharp contrast, all of the control group showed bleeding by the end of the third week of life and 7 of the 18 had died of hemorrhage by this time. All died of hemorrhage by the thirtieth day.

*Summary.* Vitamin K (potassium 2-methyl-1,4-naphthohydroquinone disulfate), injected into eggs prior to their incubation, produces vitamin K stores in newly-hatched chicks. The injected vitamin protects against the rapid and extreme fall of the prothrombin level which occurs when chicks are placed on a vitamin K-free diet.

# Vitamin K Requirement of the Newborn Infant.\*

R. L. SELLS, S. A. WALKER AND C. A. OWEN.

(Introduced by H. P. Smith.)

*From the Department of Obstetrics and Gynecology, and the Department of Pathology, State University of Iowa, Iowa City.*

During the first week of life the plasma prothrombin level of infants commonly falls to dangerously low levels.<sup>1-4</sup> The fall can be minimized or entirely prevented by prophylactic administration of vitamin K to the infant after delivery<sup>4-8</sup> or to the mother prior to delivery.<sup>4, 6</sup> When the treatment is given directly to the infant, a dosage of 1 mg of synthetic vitamin is usually employed. We agree that this is probably the proper dosage, but we shall show that it exceeds the minimal daily requirement almost 1000-fold. In view of this, we wish to suggest that a reasonable intake of milk, artificially supplied, may furnish enough vitamin K to meet the minimal needs of the normal infant, and that synthesis of the vitamin by intestinal bacteria may not be so important as commonly assumed.

In the present studies we administered 2-methyl-4-amino-1-naphthol (Synkamin) intramuscularly as a source of vitamin K activity. Treatment was of 3 types: (1) Maintenance therapy, consisting of small daily injections of the vitamin; (2) Prophylactic therapy, consisting of a single dose administered shortly after birth; (3) Curative therapy, in which case a single dose was given after depletion had already become established.

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\* Aided by a grant from the John and Mary R. Markle Foundation. Funds for assistance were also supplied by the Graduate College, State University of Iowa.

The Synkamin used in these studies was kindly furnished by Parke, Davis and Company.

<sup>1</sup> Brinkhous, K. M., Smith, H. P., and Warner, E. D., *Am. J. M. Sc.*, 1937, **193**, 475.

<sup>2</sup> Owen, C. A., Hoffman, G. R., Ziffren, S. E., and Smith, H. P., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 181.

<sup>3</sup> Quick, A. J., and Grossman, A. M., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 227.

<sup>4</sup> Waddell, W. W., and Guerry, D., *J. A. M. A.*, 1939, **112**, 2259.

<sup>5</sup> Waddell, W. W., Guerry, D., Bray, W. E., and Kelley, O. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **40**, 432.

<sup>6</sup> Hellman, L. M., and Shettles, L. B., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 138.

<sup>7</sup> Nygaard, K. K., *Acta obst. et gynec. Scandinav.*, 1939, **19**, 361.

<sup>8</sup> Dam, H., Tage-Hansen, E., and Plum, P., *Ugesk. f. laeger*, 1939, **101**, 896.

Table I shows the results of maintenance therapy with vitamin K, along with the results obtained in 6 untreated controls. In the latter, the prothrombin usually reached its lowest level on the third or fourth day. The treated cases show that within the range of 0.5-2.0  $\mu$ g of vitamin, daily, there is some variation in the response, but at 2  $\mu$ g, and at still higher levels, the fall is minimal or entirely absent.

Table II shows the effect of a single injection of the vitamin, given within the first few hours of life. When the dosage is 5  $\mu$ g or less, the prothrombin level eventually falls, but the fall is not so profound as when no vitamin is given. At the level of 10-20  $\mu$ g, the prothrombin level is reasonably stable, and when the dose is

TABLE I.  
The Daily Maintenance Requirement for Vitamin K.\*

Case No.	Daily dose vit. K $\mu$ g	Plasma prothrombin level†						
		first day %	second day %	third day %	fourth day %	fifth day %	sixth day %	seventh day %
1	0	116	74	58	36	53	80	90
2	0	88	68	38	38	41	87	96
3	0	108	42	37	31	48	81	95
4	0	102	58	32	21	40	61	98
5	0	86	58	25	27	23		‡
6	0	100	31	18	10	8		‡
7	0.05	108	83	65	60	72	100	
8	0.1	128	110	71	62	64	84	
9	0.2	114	103	75	68	80	106	
10	0.5	114	107	83	110	110	123	
11	0.5	97	103	92	100	116	123	
12	0.5	115	98	91	100	112	112	
13	0.5	75	63	73	64	82	85	‡
14	1.0	94	86	76	93	92	102	
15	1.0	115	100	103	110	112	118	
16	1.0	78	95	107	105	95	102	
17	1.0	104	92	88	93	97	102	
18	1.0	110	107	110	117	109		‡
19	1.0	87	92	95	115	100		§
20	2.0	97	83	80	90	102	112	
21	2.0	97	102	110	107	112		‡
22	2.0	108	92	95	94	93		§
23	5.0	96	89	101	111	106	100	
24	10.0	94	89	103	107	102	104	

\*Synkamin solution was diluted with normal saline to provide the indicated dosage. Injections were made into the gluteal muscles.

†Prothrombin determinations were made by a micro-adaptation of the "bedside test" (Ziffren, S. E., Owen, C. A., Hoffman, G. R., and Smith, H. P., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 595).

When glucose was given, a 10% solution was employed and a total of 200-400 cc were administered. The infants, so treated, received neither colostrum nor milk during the period of study. Those infants which received "no colostrum" were given neither sugar nor milk.

‡Glucose daily.

§No colostrum.



TABLE II.  
The Prophylactic Treatment of Infants with Vitamin K.

Case No.	Vit. K given first day only $\mu\text{g}$	Plasma prothrombin levels					
		first day %	second day %	third day %	fourth day %	fifth day %	sixth day %
25	1	98	100	89	55	78	89
26	2	108	92	57	27	57	83
27	5	96	107	93	73		
28	5	125	91	88	102	104	
29	10	103	98	95	99	94	
30	20	89	89	100	102	98	95
31	20	114	114	103	78	100	118
32	50	112	110	110	116	118	113
33	100	110	100	102	105	97	98
34	1000	97	101	108	105	103	108

Prothrombin determination and vitamin K administration were carried out as in the experiments given in Table I.

still larger the fall in prothrombin is no longer apparent. From these data it is evident that the total vitamin K requirement for the first 5 days of life is definitely higher when the vitamin is given in a single dose at the beginning of the period than when given in the form of small daily injections. The experiments show, moreover, that a certain amount of storage is possible, but that considerable "wastage" does occur.

It has been shown that the prothrombin level of the infant can be stabilized by the administration of 1 mg of the vitamin to the mother prior to delivery.<sup>9</sup> Since the prophylactic requirement of the infant is 10 to 20  $\mu\text{g}$ , it follows that at least 1-2% of the vitamin given to the mother must pass into the fetal circulation. That this much should reach the fetus is not surprising when one recalls that the weight of the infant is about 5% as great as that of the mother. One is tempted to postulate a simple physical partition of the injected vitamin between the mother and fetus.

Fig. 1 deals with curative rather than with the prophylactic dosage. The vitamin, given intramuscularly in one dose, was supplied on the second, third, or fourth day, while the prothrombin level was low. The response to 0.5  $\mu\text{g}$  or less was slow and was incomplete. At the level of 1  $\mu\text{g}$  the response was maximal, though still rather slow. With larger doses, 5 to 1000  $\mu\text{g}$  a maximal response is usually obtained in as short a period as 8 to 10 hours.

In a number of cases prothrombin determinations were made within the first 60 minutes after injection of the vitamin. However,

<sup>9</sup> Bohlender, G. P., Rosenbaum, W. M., and Sage, E. C., *J. A. M. A.*, 1941, **116**, 1763 (literature cited).

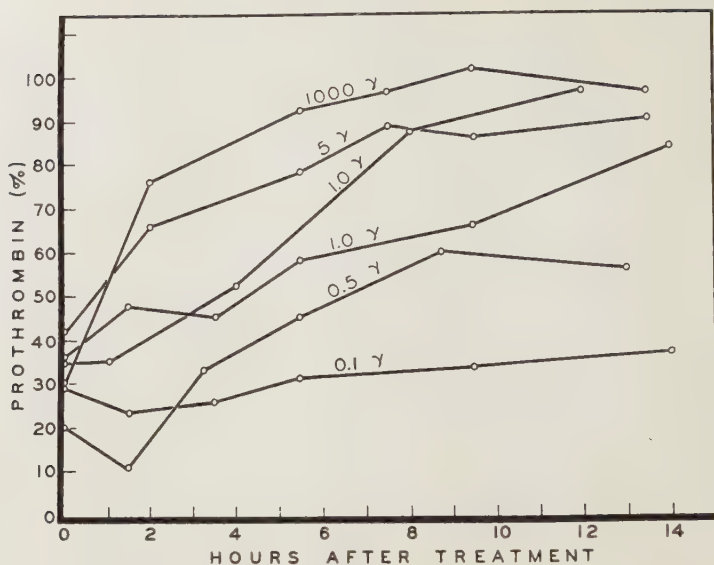


FIG. 1.

The curative dose of vitamin K.

no detectable response was observed in this short interval. The initial delay in response is evidently associated with metabolic processes, not with sluggish absorption of the vitamin from the site of intramuscular injection, for data already published<sup>10</sup> show a similar delay where the vitamin is given intravenously in 1 mg doses.

Most workers now agree that the fall in prothrombin during the first 4 days of life is soon followed by a rise. It is significant that this rise develops as soon as the supply of breast milk becomes abundant; in fact Salomonsen<sup>11, 12</sup> showed that the fall could be prevented by simply giving 30-60 cc of cow's milk during the first few days of life. Milk does not contain large amounts of vitamin K, and Salomonsen believed that the main role of the milk consists in providing a culture medium for intestinal bacteria which then produce the vitamin K in necessary quantities. Others<sup>3</sup> had already suggested the importance of bacteria, but without placing special emphasis on the role of milk. However, on finding that the vitamin K requirement of the newborn infant is extremely low, we suspected at once that the milk may of itself contain adequate amounts of preformed vitamin K. In testing this possibility we fed milk

<sup>10</sup> Willumsen, H. C., Stadler, H. E., and Owen, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 116.

<sup>11</sup> Salomonsen, L., and Nygaard, K. K., *Acta paed.*, 1939, **27**, 209.

<sup>12</sup> Salomonsen, L., *Acta paed.*, 1940, **28**, 1.

powder as a curative procedure to infants on the third or fourth day of life. We found that 5-10 g, given in water, gave a prothrombin response equivalent to 0.5-1.0  $\mu$ g of the synthetic vitamin. If the dried milk be extracted with ether, a dose several times as large is required to give the same response. The ether-soluble extract from 10-15 g of dried milk is more than adequate to give this response.

Colostrum is consumed in small quantities during the first 3 days of life, and evidence indicates that it does contain small amounts of vitamin K. In 2 cases (19 and 22 of Table I), nursing problems were such that the infants received water, but neither colostrum nor glucose. Several others, shown in Table I, received glucose, but no colostrum. There is some variation in results, but the general trend is toward lower prothrombin levels. We believe that the colostrum contains enough vitamin K to account for this small difference.

*Summary.* The vitamin K requirement of the newborn infant is shown to be extremely low (approximately 1  $\mu$ g of synthetic vitamin a day). Milk contains enough preformed vitamin K to meet this minimal requirement.

### 13169

#### Absence of Supplementary Relationships in Requirements for Pyridoxin and Essential Fatty Acids.\*

GLADYS A. EMERSON.

*From the Institute of Experimental Biology, University of California, Berkeley.*

A number of investigators<sup>1-8</sup> have been concerned with the question of a possible supplementary relation existing between pyridoxin and the essential fatty acids.

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The following materials were generously contributed: thiamin, pyridoxin and  $\alpha$ -tocopherol, Merck and Co.; riboflavin, Hoffmann-LaRoche Co.; 92% liver extract, Eli Lilly and Co.; calciferol, Mead Johnson and Co.

<sup>1</sup> Richardson, L. R., and Hogan, A. G., *Univ. of Missouri Research Bull.*, 1936, No. 241.

<sup>2</sup> Quackenbush, F. W., and Steenbock, H., *J. Biol. Chem.*, 1938, **123**, 97.

<sup>3</sup> Salmon, W. D., *J. Biol. Chem.*, 1938, **123**, 104.

<sup>4</sup> Birch, T. W., *J. Biol. Chem.*, 1938, **124**, 775.

Recent experiments<sup>9, 10</sup> have indicated that pyridoxin, pantothenic acid and linoleic acid (or arachidonic acid) are necessary for protection against dermatitis.

*Experimental.* Experiments were designed to test the possible sparing action of linoleic acid upon the pyridoxin requirement of rats and vice versa.

The diet employed differed from the usual fatty acid deficient ration in that the components of the vitamin B complex were supplied in crystalline form (except the filtrate fraction which was given as a liver filtrate). In the usual fatty acid free diet, the vitamin B complex is supplied in the form of an ether-extracted brewers' yeast. Three levels (low, intermediate and high) of an essential fatty acid (ethyl linoleate) were fed in conjunction with two levels of pyridoxin (see table I).

Female rats from different litters were segregated into the several groups. Each litter had a representation in the 3 control groups: namely, the fatty acid deficient, pyridoxin deficient, and high fatty acid-high pyridoxin (groups 1, 4 and 5). The rats were placed on diet 822 and supplements at weaning (21 days) when their average weight was 49 g.

Diet 822.	
Casein (alcohol: ether extracted <sup>11</sup> )	24
Sucrose	72
Salts (McCollum 185)	4

The following supplements were fed six times weekly:

Thiamin	15 $\mu$ g
Riboflavin	20 "
Liver filtrate <sup>12</sup>	equivalent to 5 g fresh liver†
Carotene	80 $\mu$ g (fed in 2 drops of ethyl laurate)
Calciferol	12 IU (fed in 2 drops of ethyl laurate)
$\alpha$ -tocopherol	0.5 mg (fed in 2 drops of ethyl laurate)

The two levels of pyridoxin fed were 5 and 15  $\mu$ g (the daily re-

<sup>5</sup> Quackenbush, F. W., Platz, B. R., and Steenbock, H., *J. Nutr.*, 1939, **17**, 115.

<sup>6</sup> Oleson, J. J., Bird, H. R., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1939, **127**, 23.

<sup>7</sup> Tange, U., *Sci. Papers Inst. Phys. Chem. Res., Tokyo*, 1939, **36**, 482.

<sup>8</sup> Schneider, H., Steenbock, H., and Platz, B. R., *J. Biol. Chem.*, 1940, **132**, 539.

<sup>9</sup> Richardson, L. R., and Hogan, A. G., *Proc. Am. Soc. Biol. Chem.*, 1941, 106.

<sup>10</sup> Salmon, W. D., *Proc. Am. Soc. Biol. Chem.*, 1941, 109.

<sup>11</sup> Blumberg, H., *J. Biol. Chem.*, 1935, **108**, 227.

<sup>12</sup> Lepkovsky, S., Jukes, T. H., and Krause, M. E., *J. Biol. Chem.*, 1936, **115**, 557.

† In "filtrate factor" tests the equivalent of 10 g of fresh liver stimulated growth to the same extent as did the equivalent of 5 g.

quirement of the rat appears to be approximately 10  $\mu\text{g}$ .<sup>13, 14</sup>) Three levels of ethyl linoleate, 20, 40 and 80 mg, were given in conjunction with the 2 levels of pyridoxin.

Rats receiving the fatty acid deficient diet (Group 1) exhibited the clinical symptoms of fatty acid deficiency involving a plateauing in growth, scaliness, dandruff, etc. (the classical syndrome of Burr and Burr). 20 mg of ethyl linoleate 6 times weekly (Group 2) protected against the dermatitis, but growth was stimulated to a greater extent with 40 and 80 mg than at the lower level (Groups 3 and 4).

Rats receiving the same diet with 80 mg of ethyl linoleate 6 times weekly but deficient in pyridoxin (Group 5) showed the typical picture of pyridoxin deficiency, namely, subnormal growth and acrodynia (edematous paws, thickened ears, encrusted nose, etc.). Five  $\mu\text{g}$  of pyridoxin<sup>‡</sup> did not completely prevent the appearance of the dermatitis. Little difference was apparent between the animals receiving the 3 levels of ethyl linoleate (Groups 6, 7 and 8). Growth was limited by the low pyridoxin intake as was evidenced by the weights of the animals receiving the higher level of pyridoxin.

These results indicate that the two deficiencies are independent of each other. The types of dermatitis produced are easily distinguishable. The scaliness of fatty acid deficiency was observed on a diet

TABLE I.  
Growth and Dermatitis in Relation to the Intake of Ethyl Linoleate and Pyridoxin.

Group	No. of animals	Pyridoxin $\mu\text{g}$	Ethyl linoleate mg	Gain in wt, 150 days	Type of dermatitis
1. Fatty acid deficient, pyridoxin high	12	15	—	81	Scale
2. Fatty acid low, pyridoxin high	6	15	20	124	None
3. Fatty acid intermediary, pyridoxin high	6	15	40	136	"
4. Fatty acid high, pyridoxin high	12	15	80	145	"
5. Fatty acid high, pyridoxin deficient	12	—	80	72	Severe acrodynia
6. Fatty acid low, pyridoxin low	6	5	20	124	Mild "
7. Fatty acid intermediary, pyridoxin low	6	5	40	120	" "
8. Fatty acid high, pyridoxin low	6	5	80	115	" "

<sup>13</sup> Dimick, M. K., and Schreffler, C. B., *J. Nutr.*, 1939, **17**, 23.

<sup>14</sup> Reedman, E. J., Sampson, W. L., and Unna, K., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 112.

<sup>‡</sup> Reedman and coworkers<sup>14</sup> found that 2.5  $\mu\text{g}$  of pyridoxin prevented the dermatitis over a 12-week test period.



supplemented with 15  $\mu$ g of pyridoxin while the florid dermatitis (acrodynia) characteristic of pyridoxin deficiency was readily produced on a diet supplemented with 80 mg of ethyl linoleate.

*Summary.* In an attempt to disclose the possibility of a "sparing action" of fatty acids on pyridoxin deficiency, varying levels of ethyl linoleate were fed in conjunction with a subnormal and an optimal level of pyridoxin; the "sparing action" was not observed.

### 13170

#### Failure to Cure or Prevent Graying of Rats with p-Amino Benzoic Acid.\*

GLADYS A. EMERSON.

*From the Institute of Experimental Biology, University of California, Berkeley.*

Ansbacher<sup>1</sup> has reported that p-amino benzoic acid would cure the characteristic graying of areas of the pelage of rats maintained on a vitamin B deficient diet supplemented with thiamin hydrochloride, riboflavin, pyridoxin hydrochloride, calcium pantothenate, nicotinic acid, inositol and choline chloride. On the appearance of this paper a considerable number of characteristically grayed rats were available to us for experimentation. They had been raised on a somewhat different ration than that employed by Ansbacher, for they received a vitamin B deficient diet supplemented with only three substances, to wit: thiamin hydrochloride, riboflavin and pyridoxin hydrochloride. They were divided into four groups as follows:

(1) Controls: Animals continued on the same diet and supplements which had produced the graying.

(2) Animals receiving daily, in addition to the 3 supplements mentioned, 100  $\mu$ g pantothenic acid.

(3) Animals receiving daily, in addition to the 3 supplements mentioned, 3 mg of p-amino benzoic acid.

(4) Animals receiving daily, in addition to the 3 supplements mentioned, both 100  $\mu$ g of pantothenic acid and 3 mg of p-amino benzoic acid.

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\*Aided by grants from the Research Board and the Department of Agriculture of the University of California, the Rockefeller Foundation, and Merck and Co., Rahway, New Jersey. Assistance was rendered by the Works Projects Administration, Official Project No. 65-1-08-62, Unit A-5.

<sup>1</sup> Ansbacher, S., *Science*, 1941, **93**, 164.

After 25-30 days on the diet, a marked darkening of the fur amounting practically to a cure (only "stippling" remaining) was noted in the group supplemented with calcium pantothenate alone and with calcium pantothenate plus p-amino benzoic acid. The animals receiving p-amino benzoic acid alone were not altered in appearance and were indistinguishable from the controls. There was an evident stimulus to growth as well as cure of the graying in the animals supplemented with the calcium pantothenate alone or the pantothenate and p-amino benzoic acid. The p-amino benzoic acid alone, which had no influence on the graying, also evoked no growth response.

In spite of the negative curative efforts just enumerated, there remained the possibility that graying could be produced with the exact Ansbacher diet regardless of the fact that it was supplemented with 500  $\mu$ g of calcium pantothenate daily and that such graying could be prevented or cured with p-amino benzoic acid.

Accordingly 30 animals were divided into 3 groups of 10 each, as follows: (1) This group received the Ansbacher supplements. (2) Same as above; it was planned to give p-amino benzoic acid when graying developed. (3) This group received prophylactically the Ansbacher diet plus 3 mg p-amino benzoic acid.

After more than 2 months on this diet, no evidence of graying has appeared in any of these animals. In our experience animals deficient in pantothenic acid invariably show marked graying before the expiration of this time interval.

## 13171

### Cellular Response to Insulin in Suprarenals of Pigeons.

RICHARD A. MILLER AND OSCAR RIDDLE.

*From the Carnegie Institution of Washington, Department of Genetics, Cold Spring Harbor, N. Y.*

Several years ago this laboratory reported that the suprarenals of pigeons and doves respond by enlargement to single large doses or to repeated smaller doses of insulin.<sup>1</sup> It was found further that delayed or secondary effects of heavy doses of insulin sometimes included the production of abnormally high blood sugars. Histological examination of the suprarenals indicated that the enlarge-

<sup>1</sup> Riddle, O., Honeywell, H. E., and Fisher, W. S., *Am. J. Physiol.*, 1924, **67**, 333.

ment was chiefly or wholly in the cortical tissue. Poll<sup>2</sup> confirmed this enlargement of the cortical tissue of pigeons (and rats) following insulin; in rabbits Schenk and Langecker<sup>3</sup> reported like results, while Thatcher<sup>4</sup> noted no effect. Clear and definite results with pigeons is doubtless associated with the fact that this species survives enormous doses of insulin.<sup>5</sup> In all the earlier studies on pigeons, adult birds of various races with varying weight of body and adrenals were used. We have now repeated and extended those observations on a uniform stock of young White Carneau pigeons (1.9 months from hatching), utilizing modern criteria of cellular activity in adrenal tissue, and also measured the influence of various levels of the reduced food-intake which accompanies all treatment of pigeons with insulin.

*Results.* Both before and during the present study it was known that the weights of adrenals of uninjected birds of this age and race are fairly constant. Even the fact that birds killed in autumn and winter are heavier than those of spring and summer involves a mean difference of only 9% in adrenal weight in the two groups. Thus for autumn-winter the mean weight of 62 pairs of adrenals is  $31.63 \pm .62$  mg ( $\sigma = 4.96 \pm .44$ ); for spring-summer (25 pairs of adrenals),  $28.8 \pm .89$  mg ( $\sigma = 4.45 \pm .63$ ). Though correction to uniform body weight would still further reduce this variability (and these standard errors) it does not seem advisable to make this correction for either the control or injected birds used in this study. At 10 days after hypophysectomy (anterior lobe only) the adrenals of 22 uninjected control pigeons (eating about 9 g food per bird per day) had a mean weight of  $24.00 \pm .88$  mg ( $\sigma = 4.12 \pm .62$ ). The effects on adrenal weight of insulin injection and of various degrees of underfeeding may be appraised by reference to the data just given and by the data of Table 1.

The tabulated data show that adrenal enlargement is a very regular response to single or repeated injections of insulin within a wide range of dosage. The increase in weight is sometimes small but that it is significant is attested by results of the cytological study. We have previously reported<sup>6</sup> briefly on the cytological criteria of cortical stimulation and a complete report is now in preparation. In column 6 of Table 1 the plus (+) signs indicate cortical stimulation and the minus (—) signs no stimulation. We emphasize the

<sup>2</sup> Poll, H., *Med. Klin.*, 1925, **46**, 1.

<sup>3</sup> Schenk, F., and Langecker, H., *Endocrin.*, 1935, **16**, 305.

<sup>4</sup> Thatcher, H. S., *J. Exp. Med.*, 1926, **43**, 357.

<sup>5</sup> Riddle, O., *Proc. Soc. Exp. Biol. and Med.*, 1923, **20**, 244.

<sup>6</sup> Miller, R. A., and Riddle, O., *Anat. Rec.*, 1939, **75**, 103.

TABLE I.  
Effect of Various Doses of Insulin (Lilly) on Suprarenal Weight and Activity in  
Young (1.9 mo.) Carneau Pigeons.

Test	No. of birds	Dosage		Wt, mg	Adrenal		Food given or eaten
		No. of days	Units per day		Cytology		
					Cortex	Medulla	
I	4	2	1	35.4	+	—	Fed 15 g/bird/day
II	3H*	10	1‡	47.6	+	+	Ate 0.2 " " "
III	1H	2	2‡	31.7	+?	—	Fed 10 " " "
	1H	4		37.3	+	—	
	1H	8		27.2	+	—	
IV	5H	8	4‡	45.7	+	+	" 10 " " "
V	1	2	6‡	38.5	+	—	" 10 " " "
	1	4		46.5	+	—	
	1	8		41.8	+	+	
VI	5	10	6‡	37.2	—+§	—	" 20 " " "
VII	4	2	10	32.9	+	+	" 15 " " "
VIII	3H	2	10	35.1	+	+	" 15 " " "
IX	2H	5	15	47.8	+	+	" 15 " " "
X	2	1½ hr	30	31.9	—	—	" 15 g 24 hr earlier
XI	4	10 hr	30	33.1	+	+	" 15 " " "
XII	7	1	30	37.8	+	+	" 15 " " " "
XIII	8	2	30	39.6	+	+	" 15 g/bird/day
XIV	3	3	30	40.2	+	+	" 15 " " "
XV	1	4	60	68.2	+	+	Ate no food
				Uninjected	Controls.		
A	63	(Aut.-Win.)		31.6	—	—	Ate about 35 g/bird/day
B	25	(Spr.-Sum.)		28.8	—	—	" " 30 " " "
C	5			30.7	—	—	Fed 9 g/bird/day
D	26			31.0	—	—	Complete fast, 10 days
E	22H*	(Oper. 10 days)		24.0	—	—	Ate about 9 g/bird/day
F	20H	" " "		23.8	—	—	Complete fast, 10 days

\*Hypophysectomized; anterior lobe only removed.

†Protamine zinc insulin used here.

‡The daily dose divided and given in two injections.

§Three unstimulated weighed 33.5 mg; 2 stimulated weighed 42.7 mg.

observation that the cytological changes in the cortex following insulin injection are entirely similar to those which follow the injection of a whole anterior lobe extract or an adrenotrophic fraction from pituitary tissue.

Cytological evidences of medullary activity are represented similarly in column 7 of Table I. Adrenals of control birds, after fixation in Champy and staining in anilin acid fuchsin and methyl green (our usual procedure), regularly show medullary cells which stain deep yellowish-green and are filled with closely packed chromaffin granules. A much smaller number of cells show only a light granulation and are blue-green in appearance. In some of the injected animals these lightly granulated medullary cells are more numerous and the heavily granulated cells relatively infrequent. It is also notable that the mitochondria are increased in number and the Golgi apparatus is hypertrophied, a condition which we believe

indicates increased medullary activity. Mitoses were also observed in the medullary cells following insulin. These are usually few in number, sometimes only one or two per section, but since we have never seen more than a total of one or two mitoses in hundreds of sections of adrenals from control animals this finding in insulin-treated animals is significant. Certainly, however, hyperplasia and hypertrophy are far less prominent in medullary cells than in cortical cells.

It is well known that fasting increases the sensitivity of the animal to insulin, and our data suggest that the response of the adrenal to insulin may also be somewhat modified by the amount of food consumed (Tests V, VI). Six units daily, given in 2 doses of 3 units each, effectively enlarged the adrenals at 2, 4, and 8 days in pigeons fed only 10 g of food daily; another group similarly injected for 10 days, but fed 20 g food per day, showed weight increase and cytological evidence of stimulation in only 2 birds of the group of 5. Fasting in itself has no observable effect on the weight or cytology of the adrenal (Tests D, F). Similarly the adrenals of hypophysectomized pigeons are more sensitive to insulin than are those of normals; they respond to a lower dose (Tests III, V; IV, VI) and show greater enlargement when the dosage is uniform (VII, VIII).

*Discussion.* Response of the adrenal to insulin has bearing on the question of the interpretation of the response of the adrenal to other agents. The work of others has shown that the mammalian adrenal reacts to toxic substances and various operative procedures by enlarging, one part of a syndrome which Selye has called the "alarm reaction." On Selye's view such adrenal enlargement is a secondary result of the action of the "alarming" stimulus upon the pituitary. He obtained no adrenal enlargement in hypophysectomized animals subjected to "alarming" stimuli.<sup>7</sup> In our studies cortical enlargement and loss of chromaffin granules in the medulla, duplicate changes associated with the alarm syndrome. It is entirely certain, however, that following insulin these particular changes occur in pigeons deprived of their anterior pituitary gland with the same facility as in the intact animals.

Our published<sup>8</sup> and unpublished data show that thyroxin and estrogens are effective in stimulating the adrenal in both normal and hypophysectomized pigeons. Insulin, however, stands apart from those 2 hormones in its marked ability to enlarge the adrenal, notably in the hypophysectomized pigeon. Since the adrenal is

<sup>7</sup> Selye, H., and Collip, J. B., *Endocrinology*, 1936, **20**, 667.

<sup>8</sup> Miller, R. A., and Riddle, O., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 518.



clearly involved in carbohydrate metabolism it seems more than coincidence that this gland—particularly the cortex—responds especially well to injections of insulin. The present data, and also the observation that repeated injections of huge doses of insulin progressively lose their ability to depress the blood sugar and usually soon lead to a marked hyperglycemia,<sup>1, 9</sup> suggest that both cortical and medullary parts of the adrenal actively oppose the ability of insulin to decrease the blood sugar; and that this occurs in pigeons deprived of their anterior pituitary glands.<sup>9</sup> In a recent review of pituitary-adrenal cortex relationships Swann<sup>10</sup> concludes that in carbohydrate regulation the adrenal cortex is probably completely dependent upon pituitary stimulation. The present results seem to provide an exception.

*Summary.* In tests made on 41 normal young White Carneau pigeons daily injections of 1-60 units of insulin during 10 hours to 10 days enlarged the adrenals, produced mitosis and cellular activity. These cytological effects were more pronounced in cortical cells than in medullary cells. Wholly comparable effects were produced in the adrenals of 16 additional birds previously deprived of their anterior pituitary glands. The cytological changes in the cortex following insulin are identical with those induced by adrenotropic pituitary extracts. Food consumption of the test animals and of 73 controls was regulated at different and definite levels. The relation of these results to the alarm reaction and to the rôle of the adrenals in carbohydrate regulation is discussed.

13172

# Growth of the Lobule-Alveolar System of the Mammary Gland With Pregneninolone.\*†

JOHN P. MIXNER AND CHARLES W. TURNER.

*From the Department of Dairy Husbandry, University of Missouri, Columbia, Mo.*

Turner and Frank<sup>1</sup> have shown that the growth of the lobule-alveolar system of the mammary gland which normally occurs dur-

<sup>9</sup> Riddle, O., and Opdyke, D. F., unpublished data.

<sup>10</sup> Swann, H. G., *Physiol. Rev.*, 1940, **20**, 493.

\* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 759.

† This study has been aided in part by a grant of the International Cancer Research Foundation.

<sup>1</sup> Turner, C. W., and Frank, A. H., *Missouri Agr. Exp. Sta. Res. Bul.* 174, 1932.

ing the first half of pregnancy could be stimulated experimentally in castrate animals by the simultaneous injection of estrogen and progestin. Selye<sup>2</sup> reported that the administration of progesterone to spayed female rats, not pretreated with estrin, at the rate of 15 mg per day for 15 days caused a development of the mammary lobule system similar to that seen in late pregnancy. It has been shown that the action of the ovarian hormones upon the growth of the mammary gland is mediated by the AP for these hormones are ineffective in the hypophysectomized animal<sup>3</sup> and the AP of pregnant animals contains a factor which will stimulate lobule-alveolar growth.<sup>4</sup>

Recently the writers<sup>5</sup> have developed a technic for the assay of the mammogenic lobule-alveolar growth factor in pituitary materials, which technic may also be used to assay progesterone and progesterone-like substances that stimulate the secretion of the mammogenic factor by the AP of the assay animal. The ovariectomized virgin female mouse is used as the assay animal, and a minimal development of the lobule-alveolar system of the mammary gland is required for a positive response. A mouse unit of the lobule-alveolar growth promoting substance according to this assay is defined as the total amount of material required per mouse, injected subcutaneously daily over a period of 10 days, to obtain definite lobule-alveolar development in  $50 \pm 10\%$  of 10 or more castrate, nulliparous, female mice weighing between 12 and 18 g. By this assay method 1 mg of progesterone (plus 133 i. u. of estrone) gave 60% positive responses in 10 mice.

A new synthetic hormone, pregneninolone (see 6 for references) known also as anhydro-hydroxy-progesterone and ethinyl testosterone, has been reported as having estrogenic, androgenic, and progestational properties,<sup>7</sup> the latter property being most marked and as also being orally active. The assay technic described above was used to study the ability of pregneninolone<sup>‡</sup> to stimulate mammary lobule-alveolar growth. As pregneninolone is reported to have both estrogenic and progestin-like properties it was thought that it might not need estrogen as an accompanying hormone for mammary

<sup>2</sup> Selye, H., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 343.

<sup>3</sup> Gomez, E. T., and Turner, C. W., *Missouri Agr. Exp. Sta. Res. Bul.* 259, 1937.

<sup>4</sup> Mixner, J. P., Lewis, A. A., and Turner, C. W., *Endocrinology*, 1940, **28**, 888.

<sup>5</sup> Mixner, J. P., and Turner, C. W., *Endocrinology*, in press.

<sup>6</sup> Salmon, U. J., and Geist, S. H., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 522.

<sup>7</sup> Emmens, C. W., and Parkes, A. S., *J. Endocrinology*, 1939, **1**, 332.

<sup>‡</sup> Pregneninolone was kindly supplied by Dr. E. Schwenk, Schering Corporation.

TABLE I.  
Lobule-Alveolar Responses of Castrate Female Mice to Pregneninolone Injected Subcutaneously for 10 Days.

No. of mice	Dosage in mg (total)	Responses		% positive
		Positive	Negative	
16	20-12.5	16	0	100
12	10.0	5	7	42
8	7.5	2	6	25
10	5.0-2.5	0	10	0

lobule growth. Table I presents the results secured with pregnenolone alone in promoting mammary lobule-alveolar growth.

In all injection groups propylene glycol was used as the carrier for the pregnenolone. In almost every case .2 cc per day of the propylene glycol with the pregnenolone was injected. From the table it may be seen that 10 mg of pregnenolone gave approximately a 50% response.

In another group of experiments (Table II), the effect of injecting a constant amount of an estrogen (estrone) with the pregnenolone was studied. It may be seen from a comparison of Tables I and II that approximately 1/5 as much pregnenolone is required to secure a 50% response when estrogen is administered simultaneously with the pregnenolone. Also 2.0 mg of pregnenolone (with estrone) gave 58.3% positive responses while 1.0 mg of progesterone (with estrone) as mentioned before gave 60% positive responses. Thus pregnenolone would seem to possess one-half the activity of progesterone in stimulating mammary lobule-alveolar growth when administered with a basic estrogen injection, and the activity of pregnenolone in stimulating mammary lobule-growth is enhanced 5 times by the simultaneous injection of estrogen. It is suggested that the enhancement of the growth effect is due in part to the hyperemia of genital tissues including mammary gland stroma by the estrogen. thus increasing the amount

TABLE II.  
Lobule-Alveolar Responses of Castrate Female Mice to Pregneninolone Injected Subcutaneously with Estrone for 10 Days.

No. of mice	Pregneninolone dosage in mg (total)	Estrone dosage in i.u. (total)	Results		% positive
			Positive	Negative	
12	10.0-5.0	133	12	0	100
10	2.5	133	9	1	90
12	2.0	133	7	5	58
12	1.5	133	3	9	25
12	1.0	133	1	11	8

of blood and mammogenic lobule-alveolar growth hormone available to the growing gland tissue.

*Summary.* Pregneninolone alone and injected subcutaneously with estrone into castrate virgin female mice caused the development of the lobule-alveolar system of their mammary glands, having a property similar to progesterone in this respect. The injection of estrone with the pregnenolone enhanced this activity of the pregnenolone by five times. Pregnenolone has one-half the activity of progesterone in stimulating mammary lobule-alveolar growth when both are injected with estrone.

### 13173

#### Note Concerning the Mechanism of Increased Capillary Permeability in Inflammation.

VALY MENKIN

*From the Department of Pathology, Harvard University Medical School.\**

The writer has demonstrated the presence and isolated a crystalline nitrogenous substance in inflammatory exudates which offers a reasonable explanation for the mechanism of both increased capillary permeability and migration of polymorphonuclear leukocytes at the site of inflammation.<sup>1,2</sup> This substance, termed leukotaxine, seems to belong to the group of relatively simple polypeptides. The possibility, however, of an additional prosthetic group is not yet precluded by the available data. Further studies on its chemical nature, including molecular weight, amino nitrogen content before and after hydrolysis, indole nucleus or perhaps tryptophane concentration, and a simpler method of purification will all form the subject of a separate future report.

Leukotaxine has been shown to have none of the physiological or chemical properties of histamine thus rendering difficult to accept the view that the latter plays a primary rôle in the mechanism of increased capillary permeability in inflammation.<sup>3-5</sup> The various

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\* Aided by grants from the International Cancer Foundation and the Dazian Foundation for Medical Research.

<sup>1</sup> Menkin, V., *J. Exp. Med.*, 1936, **64**, 485.

<sup>2</sup> Menkin, V., *J. Exp. Med.*, 1938, **67**, 129, 145.

<sup>3</sup> Menkin, V., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 103.

<sup>4</sup> Menkin, V., *Physiol. Rev.*, 1938, **18**, 366.

<sup>5</sup> Menkin, V., *Dynamics of Inflammation*, 1940, Macmillan Co., New York.



reasons and tests for arriving at this conclusion have been enumerated at length in previous reports and will therefore not be reiterated here.<sup>1-5</sup>

Rocha e Silva and Dragstedt have again recently raised the issue by re-asserting the original view of Bier and Rocha e Silva that histamine seems to be the principal factor involved.<sup>6, 7</sup> The type of argumentation employed is unconvincing and the data brought forward in support of their view fails to substantiate the claim of these investigators.<sup>7</sup> Their findings of histamine in various tissue extracts, the content of which is more or less paralleled with the capacity for trypan blue to accumulate in cutaneous areas treated with these extracts merely indicates that histamine is liberated alongside with countless other substances by grinding various tissues in saline. The fact that leukotaxine is also presumably formed by injuring normal tissue in the procedure of extraction is wholly ignored. As the writer has frequently pointed out, Bier, Rocha e Silva and Dragstedt consistently have abstained from isolating leukotaxine from exudates and thus have failed to repeat his studies.<sup>3-5</sup> These authors have merely worked with either untreated exudates or unpurified extracts. By this means they have been able to demonstrate the presence of histamine in their material. The writer, on the other hand, has clearly shown that the effect of the whole exudate can be completely duplicated by the nitrogenous substance, leukotaxine, recovered in turn from exudates. Minami and Inugami have recently confirmed the studies of the writer on the isolation of leukotaxine from inflammatory exudates.<sup>8</sup> Leukotaxine has now definitely been admitted by Bier not to be histamine.<sup>9</sup> The subsequent claim advanced, namely that leukotaxine releases histamine which in turn is responsible for the increased capillary permeability has not been substantiated. In the first place the writer has demonstrated that leukotaxine directly increases cellular permeability in *Arbacia ova* whereas histamine fails to do so.<sup>10</sup> In the second place, Rocha e Silva has been unable to demonstrate that kallikrein, which induces the cutaneous accumulation of trypan blue can liberate histamine.<sup>11</sup> The original type of argumentation employed by these investigators

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<sup>6</sup> Bier, O., and Rocha e Silva, M., *Arqu. d. Inst. Biol.*, 1938, **9**, 109.

<sup>7</sup> Rocha e Silva, M., and Dragstedt, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 303.

<sup>8</sup> Minami, G., and Inugami, K., *Transact. Soc. Path. Japonicae*, 1940, **30**, 389.

<sup>9</sup> Bier, O., and Rocha e Silva, M., *Proc. Third Internat. Congress for Microbiology*, 1939, published 1940, 767.

<sup>10</sup> Menkin, V., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 588.

<sup>11</sup> Rocha e Silva, M., *Nature*, 1940, **145**, 591.



on the basis of these new findings can, therefore, be now reversed. One might just as well assert that histamine acting as any other irritant in optimum concentration releases leukotaxine, which in turn is responsible for increased capillary permeability. Finally, it is interesting to note that Rocha e Silva and Dragstedt cite the studies of Duthie and Chain in an endeavor to explain in their experiments the discrepancy occasionally encountered between the positive trypan blue test and the histamine equivalent. Duthie and Chain have confirmed the earlier studies of the writer by showing that protein degradation products obtained by enzymatic hydrolysis yield biological effects similar to leukotaxine.<sup>12, 13</sup> Since leukotaxine seems precisely to be such a by-product of protein catabolism, the fact that a positive trypan blue test has been elicited from protein breakdown products obtained by enzymatic hydrolysis is not in the least surprising. The facts merely indicate that the argument advanced on the basis of Duthie and Chain's work in no way supports a non-specific interpretation for the positive trypan blue test whenever the desired histamine equivalent in the material studied is absent.

The present series of experiments were devised in order to determine whether leukotaxine or histamine is the essential constituent which imparts to the whole exudate the property of inducing in rabbits a positive cutaneous trypan blue test. Leukotaxine or the permeability factor diffuses through a cellophane membrane by dialyzing the whole exudate against distilled water. Whatever histamine is present in exudates presumably also diffuses outward by such procedure. Exudates were therefore dialyzed for about 18 to 20 hours against distilled water. The cellophane bag during dialysis was continuously rotated by having it attached to a stirring apparatus. The protein material which remained behind in the cellophane tube was found, when introduced into the skin of a rabbit, incapable of inducing the characteristic pattern of accumulation of trypan blue from the circulation. This is in complete agreement with earlier studies.<sup>1</sup> To this inactive residual fraction of the dialyzed exudate, leukotaxine is now added in concentration ranging from 1 to 3 mg. The mixture is then injected into the skin of a rabbit. The dye accumulates rapidly from the blood stream in such cutaneous areas. The pattern is in general no different from that elicited by the undialyzed exudate. Thus, by the addition of leukotaxine, the capacity of the dialyzed residual exu-

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<sup>12</sup> Duthie, E. S., and Chain, E., *Brit. J. Exp. Path.*, 1939, **20**, 417.

<sup>13</sup> Menkin, V., *J. Exp. Med.*, 1938, **67**, 153.

TABLE I.  
Comparison of the Effect on Capillary Permeability of Leuko taxine and Histamine Added to Exudates after Dialysis.\*

Rabbit No.	Exudate	Residual exudate after dialysis	Leukotaxine in saline, or 0.2% saline, or H <sub>2</sub> O		Nature of the fractions Tested			
					Leukotaxine + residual exudate after dialysis	Histamine 1:20,000-1:25,000 in .85% saline, or 0.2% saline, or distilled H <sub>2</sub> O	Histamine 1:20,000-1:25,000 + residual exudate after dialysis	Histamine 1:40,000-1:50,000 in .85% saline, or 0.2% saline, or distilled H <sub>2</sub> O
21-05	+++ throughout	0 in center, zone +++ at periphery	+++ throughout	+++ to +++ throughout		—		Histamine 1:40,000-1:50,000 in .85% saline, or 0.2% saline, or distilled H <sub>2</sub> O
21-22	++ throughout	0 in center, zone + at periphery	+++ to +++ throughout	++ almost throughout		—		0 in center, zone +++ at periphery
21-43	trace throughout	0 in center, zone of trace at periphery	++ throughout	++ throughout		trace throughout	0 in center, zone of trace at periphery	0 in center, zone of + at periphery
21-45	++ throughout	0 in center, zone of faint trace at periphery	+++ throughout	+++ throughout		0 throughout	0 in center, zone of + at periphery	0 in center, zone of + at periphery

\*The exudates were obtained either from dogs one day after an intrapleural injection of 1.5 cc of turpentine, or from rabbits a day after injecting 0.25 cc of 5% croton oil in olive oil into the pleural cavity. The degree of accumulation of trypan blue in treated cutaneous areas of rabbits is gauged by the number of plus signs. About 15 cc of approximately 1% trypan blue in saline was injected into the ear vein immediately after introducing the various fractions into the skin of the abdomen.<sup>1,2</sup> Final readings as indicated in the table were recorded after an interval of approximately 30 minutes to 1 hour. In the case of rabbits 21-43 and 21-45 the exudate and the dialyzed exudate were diluted 1:1 with physiological saline in order to control for the dilution factor which occurred when either leukotaxine or histamine were added 1:1 to the dialyzed exudate fraction. This, however, as indicated failed to alter the results.

dative material to induce an increased capillary permeability has been fully restored. When the same procedure is repeated with histamine dihydrochloride in concentrations of 1:20,000 to 1:50,000 which, incidentally, according to Bier and Rocha e Silva,<sup>6</sup> are the concentrations of histamine recovered from exudates, the dye usually fails to accumulate homogeneously in the skin as it does in the case of the original exudate or of leukotaxine. Thus, the exact effect on capillary permeability of the exudate is not restored. Histamine has failed to reconstitute the capacity of the exudate when added to the inactive residual exudate fraction obtained after dialysis. This, however, is accomplished perfectly well by leukotaxine or even by the dried diffusate fraction from the dialyzed exudate.<sup>1</sup> The observations are conveniently assembled in Table I.

*Conclusions.* An inflammatory exudate readily induces an increased capillary permeability as evidenced by the rapid accumulation of trypan blue from the circulation into cutaneous areas treated with the exudative material. Dialysis of the exudate allows the outward diffusion of leukotaxine. The indiffusible residual material remaining after dialysis of the exudate is essentially incapable of increasing capillary permeability with the characteristic homogeneous pattern. The exact original effect of the exudate, however, can be readily reconstituted by merely adding leukotaxine to the indiffusible fraction of the dialyzed exudate. This cannot be obtained by adding histamine in the concentration in which the latter is recovered from exudates. These facts therefore add further support to previous observations that leukotaxine and not histamine is the primary factor concerned in the mechanism of increased capillary permeability in inflammation.

### 13174

#### **Effect of Posture on Circulating Blood Volume in a Case of Orthostatic Hypotension and Tachycardia.**

PHILLIP HALLOCK AND GERALD EVANS.

*From the Division of Internal Medicine, University of Minnesota Hospitals, Minneapolis, Minn.*

It has been shown<sup>1</sup> that when an individual assumes the erect posture, a decrease in plasma volume follows. This has been explained as due to the augmented hydrostatic pressure in the vascular

bed of the lower part of the body, causing an increased capillary pressure and in turn an increased filtration of fluid from the blood stream. In individuals with orthostatic hypotension and tachycardia, it has been suggested that the abnormal drop of arterial blood pressure and the increase in pulse rate which occur upon standing result from a much greater amount of pooled blood in the lower part of the body as compared to that which occurs in normal subjects.<sup>2</sup> This theory for the most part has been based on experiments in which blood pressure and pulse rate readings were made in patients with orthostatic hypotension and tachycardia in the recumbent and erect positions. Thus, it was demonstrated that when tourniquets were applied to the proximal portion of the thighs and these patients were then placed in the upright position, the arterial blood pressure was maintained at a much higher level than without them.

This explanation has been brought into doubt recently by Stead and Ebert<sup>3</sup> who have shown that patients with orthostatic hypotension do not pool more blood in the lower extremities on standing than do normal subjects under similar conditions and that the fundamental disturbance is the loss of reflex vasoconstriction in response to the fall of blood pressure. Their conclusions were drawn from studies in which the quantities of blood contained in the legs in the recumbent and standing positions were measured in normal subjects and in subjects with orthostatic hypotension.

MacLean and Allen<sup>4</sup> have suggested that the defect in orthostatic hypotension and tachycardia was probably not a fault in the mechanism which produces reflex vasoconstriction of the arterioles but rather a defect in the maintenance of an adequate return of venous blood to the heart as evidenced by the Flack test.<sup>5</sup>

Regardless of the explanation offered, it was thought to be of value to determine in a case of orthostatic hypotension and tachycardia the extent to which the blood volume diminished upon standing and to compare this to similar observations upon normal subjects.

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<sup>1</sup> (a) Thompson, W. A., Thompson, P. K., and Dailey, M. E., *J. Clin. Invest.*, 1928, **5**, 573; (b) Waterfield, R. L., *J. Physiol.*, 1931, **72**, 110; (c) Keys, A., and Butt, H. R., *Arch. Int. Med.*, 1939, **63**, 165; (d) Turner, A. H., Newton, M. I., and Haynes, F. W., *Am. J. Physiol.*, 1930, **94**, 507.

<sup>2</sup> (a) Bradbury, S., and Eggleston, C., *Am. Heart J.*, 1925, **1**, 73; (b) Allen, E. V., and Magee, H. R., *Med. Clin. N. Amer.*, 1934, **18**, 585; (c) Ellis, L. B., and Haynes, F. W., *Arch. Int. Med.*, 1936, **58**, 773.

<sup>3</sup> Stead, E. A., Jr., and Ebert, R. V., *Arch. Int. Med.*, 1941, **67**, 546.

<sup>4</sup> MacLean, A. R., and Allen, E. V., *J. Am. Med. Assn.*, 1940, **115**, 2162.

<sup>5</sup> Flack, Martin, *Brit. Med. J.*, 1923, **2**, 921.



*Experiments.* Two normal subjects and a subject with orthostatic hypotension and tachycardia were studied. They were kept in the recumbent position overnight and on the following morning while still in the recumbent position, their blood volume, hematocrits, plasma proteins, and hemoglobins were determined. They were then placed in the upright position and kept standing motionless for a period of 10 minutes. The blood studies were then repeated at 5 and 10 minute intervals on standing. The plasma volume was estimated by the Gibson and Evans method<sup>6</sup> as adapted to the Evelyn photoelectric microcolorimeter<sup>7</sup> and the value corrected for the loss of dye from the disappearance curve of the dye from the blood stream. The plasma proteins were estimated by the Barbour and Hamilton method.<sup>8</sup> The hemoglobin concentration in grams per 100 cc was determined with the Evelyn photoelectric colorimeter<sup>9</sup> and the hematocrit percentage was determined with Wintrobe hematocrit tubes.

*Results.* When 2 normal subjects were placed in the upright position, it will be noted (Table I) that slight hemoconcentration occurs as shown by the increase of hemoglobin, hematocrit, and plasma proteins and that these are in agreement with the decrease of 80 cc of plasma volume which occurred in each case. The plasma volume differences observed in these two normal subjects are small and are reported to show the contrast with the situation found in the pathological subject. These changes, however, are in excellent agreement with those found by Thompson and Thompson,<sup>1a</sup> and Waterfield<sup>1b</sup> in their studies on the effect of upright posture on changes in plasma volume in normal subjects.

When the patient with orthostatic hypotension and tachycardia was placed in the upright posture, a remarkable exaggeration of the normal filtration process was observed (Table II). It should be stated that when this patient was tilted in the upright position, his blood pressure dropped very quickly to 50 mm of Hg. and his pulse rate rose to 150. Although he felt very weak and complained of blurring of vision, there was no loss of mental acuity and he was able to maintain himself in the upright position for 10 minutes without fainting. It will be noted that on standing for 10 minutes there was an absolute decrease of 576 cc (11%) in plasma volume from the resting value and a diminution of 650 cc, or 7½%, of whole blood from the resting value. That this is not due to a mere

<sup>6</sup> Gibson, J. G., and Evans, W. A., Jr., *J. Clin. Invest.*, 1937, **16**, 301.

<sup>7</sup> Gibson, J. G., and Evelyn, K. A., *J. Clin. Invest.*, 1938, **17**, 153.

<sup>8</sup> Barbour and Hamilton, *J. Biol. Chem.*, 1926, **69**, 625.

<sup>9</sup> Evelyn, K. A., *J. Biol. Chem.*, 1936, **115**, 63.



TABLE I.  
Effect of Erect Position on Blood Volume, Hematocrit, Plasma Proteins and Hemoglobin in Two Normal Adults.

Position	Blood pressure	Pulse rate	Hemoglobin, g	Hematocrit, %	Plasma proteins, g	Plasma vol., cc	Total blood vol., cc
Case 1							
Recumbent	105/75	72	15.44	51.5	6.63	2,910	6,000
After standing (10 min)	100/75	80	15.89	52.0	7.07	2,830 (diff. 80)	5,900 (diff. 100)
Case 2							
Recumbent	135/79	64	14.57	45.0	8.34	2,480	4,510
After standing (5 min)	110/78	84	15.12	46.2	8.68	2,400 (diff. 80)	4,460 (diff. 50)

pooling of the blood in the vascular bed of the lower part of the body but rather to an augmented filtration into the tissue spaces is supported by the significant rises in the hemoglobin concentration, plasma proteins, and hematocrit readings. In each instance, there is evidence of hemoconcentration; namely, an increase of 0.8 g (5%) in hemoglobin, increase of 6.5% in hematocrit, and an increase of 0.83 g (14%) in plasma proteins. Emphasis is laid upon the fact that in a general way the various measurements, each indicating hemoconcentration, are in agreement with each other.

It may not be amiss to point out that the lower extremities are not necessarily the most important blood reservoirs for a pooling effect but that the splanchnic area is quantitatively very important and may be determining.

*Conclusion.* Evidence is presented that abnormally great hemoconcentration occurs in the erect posture in a subject with orthostatic hypotension and tachycardia. The magnitude of the effect is great enough to permit the conclusion that abnormal filtration rates aggravate the circulatory embarrassment in this condition.

TABLE II.  
Effect of Erect Position on Blood Volume, Hematocrit, Plasma Proteins, and Hemoglobin in Individual with Orthostatic Hypotension and Tachycardia.

Position	Blood pressure	Pulse rate	Hemoglobin, g	Hematocrit, %	Plasma proteins, g	Plasma vol., cc	Total blood vol., cc
Recumbent	104/66	90	15.2	51.5	5.67	4,120	8,490
Standing (10 min)	50/?	150	16.0	54.8	6.5	3,544 (diff. 576)	7,840 (diff. 650)
Two weeks later							
Recumbent	105/70	80		45.		4,280	7,782
Standing (10 min)	52/?	140		48.5		3,690 (diff. 590)	7,235 (diff. 547)

### Production of Cardiac Hypertrophy in Rats.

GEORGE HERRMANN, GEORGE DECHERD AND PETER ERHARD.

*From the Department of Medicine, University of Texas School of Medicine,  
Galveston*

In this laboratory we have been interested for some years in the problem of the pathogenesis and production of myocardial hypertrophy.<sup>1</sup> We have employed experimental aortic valvular lesions for this purpose, but this method has been unsatisfactory, not only because of individual differences in the response of the heart, but because the individual valvular lesions were inevitably quite different in extent. We also needed a method which was applicable to large series of small animals. For these reasons we have investigated the effect of renal ischemia in the rat, employing several of the methods that have been described for impairing the renal blood supply.

The results of our various studies are shown in Table I. We first employed the technic described by Collins,<sup>2</sup> and used also by Rytand,<sup>3</sup> in which the renal artery is tied over a wire of known gauge. For rats weighing from 150 to 200 g we used a wire of B. & S. gauge No. 26. A few of these animals showed enlarged hearts, but the results were neither striking nor consistent. We also attempted the use of the procedure introduced by Page<sup>4</sup> for yielding a perinephritis by enveloping the kidney in a cellophane capsule. This, too, was unsuccessful in our rats, and in most instances the kidney with the cellophane covering seemed to act as a foreign body, and eroded its way into the stomach, leading shortly to the death of the animal. Deep Roentgen radiation over the kidney as described by Hartman<sup>5</sup> was also employed in a smaller series of animals, but the hearts of none of these rats were hypertrophied. 10,000 unit doses of Vitamin D\* were injected intraperitoneally into each of another series of rats because of the report<sup>6</sup> that such a procedure produced arteriosclerosis and hypertension. However, no cardiac hypertrophy resulted.

The molded gauze-collodion method was attended with uniform success. This method was demonstrated to us by Dr. John Williams

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<sup>1</sup> Herrmann, G., and Dechard, G. M., *Ann. Int. Med.*, 1939, **13**, 794.

<sup>2</sup> Collins, D. A., *Am. J. Physiol.*, 1936, **116**, 616.

<sup>3</sup> Rytand, D. A., *J. Clin. Invest.*, 1938, **17**, 391.

<sup>4</sup> Page, I. H., and Graef, I., *Am. J. Path.*, 1939, **15**, 623 (proc.).

<sup>5</sup> Hartman, F. W., *ibid.*, 1939, **15**, 623 (proc.).

\* Supplied by Upjohn Co.

<sup>6</sup> Ham, A. W., *Arch. Path.*, 1940, **29**, 731 (proc.).

of the Vanderbilt University Medical School. The left kidney, after being exposed and delivered through a posterior lumbar incision, was snugly wrapped with a single layer of ordinary surgical gauze. The gauze was then painted with U.S.P. collodion. As this dried another layer of collodion was applied, so that a firm molded capsule about the kidney resulted. This was not allowed to press on the renal pedicle. About 4 to 7 days after this procedure, the right kidney was removed, again through a lumbar incision. The animals tolerated this procedure well, and in our experience virtually every one that survived the immediate operative mortality of about 20% showed a conspicuous cardiac hypertrophy.

Table I shows the heart weight:body weight ratios, and the statistical constants derived from them, of each series of animals. In Table II there is a further analysis of the rats in whom renal ischemia was produced by the molded gauze-collodion capsule. We have assumed that hypertension resulted after the removal of the normal kidney. Rats sacrificed 10 days after the nephrectomy showed definite cardiac hypertrophy. The data indicate that within 30 days probably the maximum hypertrophy is obtained, although there seems to be a slight tendency towards a further increase up to about the 50th day.

*Summary.* The production of renal ischemia by molded gauze-collodion capsules, as demonstrated to us by Dr. John Williams, appears to be the most satisfactory method of producing cardiac hypertrophy in rats.

TABLE I.  
Heart Weight : Body Weight Ratios of Rats with Various Types of Renal Ischemia.

	No. of animals	HW/BW mean	P.E. mean	Stand. Dev.	Diff. $\pm$ P.E.	Diff.
Normal <sup>1</sup>		3.37	.06	.52		
Renal artery constricted	25	3.81	.09	.67	+ .44 $\pm$ .11	
X-ray	14	3.15	.08	.45	— .22	.10
Cellophane	50	3.68	.08	.88	+ .31	.10
Vitamin D	150	3.14	.05	.95	— .23	.08
Gauze-Collodion	54	5.99	.13	1.37	+2.62	.14

TABLE II.  
Percent Hypertrophy of Rat Hearts by Gauze-Collodion Method.

Time after nephrectomy	No. of animals	Range of hypertrophy, %	Avg hypertrophy, %
10-19 days	11	29- 67	46
20-29 "	6	23-106	62
30-39 "	7	61-119	82
40-49 "	10	53-137	92
50-59 "	4	42-155	90
60-69 "	15	23-130	69

Reduction of p-Nitrobenzenesulfonamide and of Azobenzene-4,4'-disulfonamide by Animal Tissues.\*

LAURA M. FLYNN AND MATHIAS F. F. KOHL. (Introduced by P. A. Shaffer.)

*From the Departments of Biochemistry and Pharmacology, School of Medicine, Washington University, St. Louis, Mo.*

The fate in the animal body of p-nitrobenzenesulfonamide, the nitro-compound corresponding to sulfanilamide, is of interest because of the sensitivity of microorganisms to this substance. Mayer and Oechsli<sup>1</sup> found it to have high antistreptococcal activity *in vivo*, and recently Burton, McLeod, McLeod and Mayr-Harting<sup>2</sup> have reported marked sensitivity of organisms of the Neisseria, Bacterium and Clostridium groups to it *in vitro*. The question arises whether the nitro-compound is effective as such or in consequence of its reduction to sulfanilamide or intermediate products.

As a contribution to this question we have studied the reduction of p-nitrobenzenesulfonamide *in vitro* by rat liver, and the extent of its reduction *in vivo* by normal rats. The reduction of azobenzene-4,4'-disulfonamide, an oxidation product of sulfanilamide, has also been investigated.

*In vitro experiments.* Fresh liver was ground with sand and water and squeezed through cheese cloth. To this suspension was added an equal volume of M/10 PO<sub>4</sub> buffer, pH 7.5, in which p-nitrobenzenesulfonamide had been dissolved. Each 5 cc of the liver suspension, as incubated, contained 2.5 mg of p-nitrobenzene sulfonamide and represented 1.25 g of liver. Two drops of toluene were added as a preservative. Aerobic incubation at 37.5°C was carried out in 25 cc Erlenmeyer flasks with cotton stoppers. Anaerobic incubation was carried out in Thunberg tubes immersed in a water bath at 37.5°C after evacuation with a Cenco Hyvac pump. Separate samples were used for analyses at various time intervals. Proteins were precipitated with 10% trichloroacetic acid and the amounts of

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\* This investigation was aided by a grant to Professor P. A. Shaffer from the Rockefeller Foundation. We are indebted to the Abbott Laboratories for the p-nitrobenzenesulfonamide and to our colleague Mr. Louis Berger for the azobenzene-4,4'-disulfonamide.

<sup>1</sup> Mayer, R. L., and Oechsli, C., *Compt. Rend.*, 1937, **205**, 181.

<sup>2</sup> Burton, H., McLeod, J. W., McLeod, T. S., and Mayr-Harting, A., *Brit. J. Exp. Path.*, 1940, **21**, 288.

TABLE I.  
Reduction of p-Nitrobenzenesulfonamide to Diazotizable Compounds by Liver Suspension.

Exp. No.	% reduction							
	Aerobic				Anaerobic			
	2 hr	4 hr	6 hr	8 hr	2 hr	4 hr	6 hr	8 hr
1		30.1		35.0		43.7		51.7
2	10.2		17.2		13.7		23.0	
3	21.2	27.6		28.5	22.5	32.4		42.8

diazotizable material in the filtrates were determined by Marshall's method.<sup>3</sup>

Reduction of p-nitrobenzenesulfonamide by rat liver brei proceeds rapidly as shown in Table I. Aerobic reduction was somewhat slower than reduction in the absence of air by the same liver brei. The rate and extent of reduction varied in different experiments but as much as one-fifth (0.4 mg per g of liver) was reduced in 2 hours, and up to one-half (1.0 mg per g) in 8 hours' incubation.

*In vivo experiments.* The p-nitrobenzenesulfonamide, suspended in 5% acacia, was administered by stomach tube to normal rats weighing about 300 g. The animals were in individual cages and the urine was collected under toluene. Urine samples collected at intervals were analyzed for free and conjugated amine by Marshall's method.<sup>3</sup> Urine samples were analyzed for total amine after reduction with zinc and hydrochloric acid. This determination includes unchanged p-nitrobenzenesulfonamide and possible intermediates such as nitroso-, hydroxylamino-, hydrazo-, azoxy-, or azo-compounds in the urine. To 10 cc of diluted urine were added 2 cc of 4N-HCl and 200 mg of granulated zinc. The sample was heated in boiling water until the zinc had dissolved. This required over an hour; more acid was added if necessary. After adjusting the pH to 1 or 2, the sample was brought back to volume and analyzed by Marshall's method. Similar treatment of known amounts of p-nitrobenzenesulfonamide gave from 94 to 104% of the theoretical yield of sulfanilamide. Corrections were made for the small amount of diazotizable material present in the urine of normal rats, about 0.1 mg per day, calculated as sulfanilamide.

Data from typical experiments are shown in Table II. From 82% to 83% of the nitrobenzenesulfonamide administered was recovered in the urine in 3 days, most of the excretion occurring during the first day. Of the total amount recovered 10%

<sup>3</sup> Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.



was free amine, 74% to 85% was conjugated amine, and 16% to 5% was excreted in a state of oxidation above the amine. In contrast to these results with p-nitrobenzenesulfonamide, the excretion of the conjugated amine by rats after sulfanilamide dosage is comparatively low. Marshall found the average 8-hour urinary excretion of sulfanilamide by rats to be 48.6% of the oral dose. Only 25% of the excreted sulfanilamide was conjugated.<sup>4</sup> It seems very possible that when nitrobenzenesulfonamide is administered conjugation occurs before reduction has reached the stage of the free amine. It is of interest to note that acetylsulfanilamide, once formed, is deacetylated to only a small extent.<sup>5-8</sup>

It seemed of interest to compare the *in vivo* reduction of an azo-compound with that of the nitro-compound. Crystalline azobenzene-4,4'-disulfonamide was prepared in this laboratory by Mr. Louis Berger by oxidation of sulfanilamide with molybdicyanide. Because of the very low solubility of the azo-compound in water, doses were given as a fine aqueous suspension, injected subcutaneously.

Data from a typical experiment are given in Table III.

TABLE II.  
Reduction of p-Nitrobenzenesulfonamide to Diazotizable Compounds by Rats.

Dose,* mg	% of dose recovered	Days	Free amine, mg	Total amine after hydrolysis, mg	Total amine after reduction, mg	Distribution of compounds recovered		
						Free amine, %	Conju- gated amine, %	Not re- duced to amine in animal body, %
20	81.7	1	.985	8.26	10.15			
		2	.353	2.93	3.14			
		3	.054	0.48	0.62			
			1.392	11.67	13.91	10.0	73.9	16.1
10	82.5	1	.60	5.03	5.25			
		2	.11	1.4	1.5			
		3	.01	0.25	0.28			
			.72	6.68	7.03	10.2	84.8	5.0

\* 10 mg of nitro-compound equivalent to 8.516 mg of amine.

<sup>4</sup> Marshall, E. K., Jr., and Cutting, W. C., *Bull. Johns Hopkins Hosp.*, 1938, **63**, 328.

<sup>5</sup> Marshall, E. K., Jr., Cutting, W. C., and Emerson, K., Jr., *J. Am. Med. Assn.*, 1938, **110**, 252.

<sup>6</sup> Ockerblad, N. F., and Carlson, H. E., *J. Urol.*, 1939, **41**, 801.

<sup>7</sup> Allen, J. G., *New Eng. J. Med.*, 1940, **222**, 1029.

<sup>8</sup> Kohl, M. F. F., and Flynn, L. M., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 455.

TABLE III.

Reduction of Azobenzene-4,4'-disulfonamide to Diazotizable Compounds by Rats.

Dose,* mg	% of dose recovered	Days	Distribution of compounds recovered				
			Free amine, mg	Total amine after hydrolysis, mg	Total amine after reduction, mg	Free amine, %	Conju- gated amine, %

10	14.5	1	.065	.155	.282			
		2	.020	.035	.104			
		3	.095	.093	.238			
		4	.110	.142	.260			
		5	.020	.020	.147			
		6	.110	.125	.440			
			.420	.570	1.471	28.5	10.2	61.3

\* 10 mg of azobenzene-4,4'-disulfonamide equivalent to 10.12 mg sulfanilamide.

Only 14% of the azobenzene-4,4'-disulfonamide injected had been recovered when urine collections were discontinued at the end of 6 days. Probably the slow excretion paralleled slow absorption from subcutaneous tissues. Of the total amount recovered 29% was free amine, 10% conjugated amine and 61% was excreted in a state of oxidation above the amine.

*Summary.* p-Nitrobenzenesulfonamide is rapidly reduced by rat liver suspensions. After oral administration p-nitrobenzenesulfonamide is rapidly reduced by rats; a high percentage of the dose is excreted in the reduced form, and the extent of conjugation of the reduced form is large. A small amount of the drug is excreted in a state of oxidation above that of the amine. Azobenzene-4,4'-disulfonamide administered subcutaneously to rats is excreted slowly, reduced to only a small extent and conjugated to only a low degree.

## Reduction of Isomeric Nitrobenzoic Acids by Rats.\*

MATHIAS F. F. KOHL AND LAURA M. FLYNN. (Introduced by  
Carl F. Cori.)

*From the Departments of Pharmacology and Biochemistry, School of Medicine,  
Washington University, St. Louis, Mo.*

Mayer and Oechslin<sup>1</sup> demonstrated that p-nitrobenzoic acid has a bacteriostatic effect upon pneumococci. p-Aminobenzoic acid, the reduction product of the nitro-compound, has been shown by Woods,<sup>2</sup> Selbie,<sup>3</sup> Rubbo and Gillespie,<sup>4</sup> Janeway,<sup>5</sup> and others to inhibit the bacteriostatic effect of sulfanilamide. It is of interest that Miller found in experiments with *Streptococcus viridans* that p-aminobenzoic acid also inhibits the bacteriostatic effect of p-nitrobenzoic acid.<sup>6</sup> Sherwin and Hynes<sup>7</sup> investigated the metabolism of the isomeric nitrobenzaldehydes in man, dog and rabbit, and recovered crystalline compounds from the urine. They concluded that the aldehydes were oxidized to the corresponding acids but found no reduction of the nitro- group.

We have studied the reduction of the isomeric nitrobenzoic acids *in vitro* by liver and kidney tissues. We have also investigated the reduction of these compounds *in vivo* by rats after oral or intraperitoneal administration.

*In Vitro Experiments.* Preparation and incubation of tissue suspensions were carried out as in previous experiments on the reduction of p-nitrobenzenesulfonamide.<sup>8</sup> Solutions of the isomeric nitrobenzoic acids were neutralized with NaOH. Each 10 cc of brei, corresponding to 2.5 g of tissue, contained 2 mg or 5 mg of substrate and M/10 PO<sub>4</sub> buffer, pH 7.5. Aliquot portions of the suspension were removed at intervals during the incubation period for analysis. Proteins were precipitated with 10% trichloroacetic

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\* This investigation was aided by a grant to Professor P. A. Shaffer from the Rockefeller Foundation.

<sup>1</sup> Mayer, R. L., and Oechslin, C., *Arch. intern. pharmacodynamie*, 1939, **62**, 211.

<sup>2</sup> Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

<sup>3</sup> Selbie, F. R., *Brit. J. Exp. Path.*, 1940, **21**, 90.

<sup>4</sup> Rubbo, S. D., and Gillespie, J. M., *Nature*, 1940, **146**, 838.

<sup>5</sup> Janeway, C. A., *J. Am. Med. Assn.*, 1941, **116**, 941.

<sup>6</sup> Miller, J. K., *J. Pharm. and Exp. Ther.*, 1941, **71**, 14.

<sup>7</sup> Sherwin, C. P., and Hynes, W. A., *J. Biol. Chem.*, 1921, **47**, 297.

<sup>8</sup> Flynn, L. M., and Kohl, M. F. F., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 466.

acid and the amounts of diazotizable material in the filtrates were determined by the use of the Marshall sulfanilamide test reagents.<sup>9</sup> The ortho-isomer coupled so slowly that readings for this compound were made three hours after addition of the reagents. Full color development required an hour with the meta-isomer. Colorimetric readings could be made fifteen minutes after addition of the reagents to the para-isomer.

Results of typical tissue experiments are shown in Table I. The reduction of ortho-nitrobenzoic acid to diazotizable, free amino-compound is accomplished to only a small extent by tissue suspensions. Meta- and para-nitrobenzoic acids are reduced readily to diazotizable compounds by suspensions of rat or mouse livers and by rat kidney.

*In Vivo Experiments.* The isomeric nitrobenzoic acids were administered orally or intraperitoneally as aqueous solutions, neutralized with NaOH, to rats that weighed about 300 g. The animals were kept in individual cages, and the urine was collected under toluene. Urine samples collected at intervals were analyzed for free and conjugated amine, by the use of Marshall's reagents, as described earlier in this paper. Urine samples were analyzed for total amino-compounds after reduction with zinc and hydrochloric acid by methods previously described.<sup>8</sup> This determination included unchanged nitro-compound and possible intermediate reduction products. Similar treatment of known amounts of the isomeric nitrobenzoic acids gave the following yields of diazotizable compounds: o-nitrobenzoic acid, 50% to 70% of the theoretical yield; m-nitrobenzoic acid, 84% theoretical; p-nitrobenzoic acid, 94% to 102% theoretical. In all

TABLE I.  
Reduction of Isomeric Nitrobenzoic Acids to Diazotizable Compounds by Tissue Suspensions.

Exp. No.	Isomer tested	Substrate concentration mg%	Tissue	% substrate changed				
				2 hr	4 hr	6 hr	7 hr	8 hr
1	Ortho	50	Rat liver	0.65	0.97		1.43	
2	"	20	Mouse liver	0.86	2.83	2.83		
1	Meta	50	Rat liver	4.8	12.4		30.3	
2	"	20	Mouse liver	7.0	12.2	23.5		
3	"	20	Rat kidney	5.2	12.0		20.4	
1	Para	50	Rat liver	9.1	14.7		28.2	
2	"	20	Mouse liver	9.9	18.0	23.2		
4	"	50	Rat kidney	3.0				10.6

<sup>9</sup> Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

experiments corrections were made for the small amount of diazotizable material excreted in the urine of normal rats.

Data from typical experiments are shown in Table II. Percentages of injected or ingested drugs recovered in the urine were as follows: ortho-isomer, 72%; meta-isomer, 63%; para-isomer, 89-94%. Excretion of the para-isomer was complete in 48 to 72 hours. Small traces of the ortho- and meta-compounds were still present in the urine after 96 hours. 88% of the total ortho-compound recovered was excreted in the first 24 hours; 80% was excreted within 8 hours. 21% of the recovered ortho-compound was excreted as free amine; amounts of conjugated amine and higher oxidation products could not be determined without altering the method used. 84% of the total meta-compound recovered was excreted in the first 24 hours; 80% was excreted within 8 hours. Of the total amount of meta-isomer recovered 8% was free amine, 22% was conjugated amine, and 70% was excreted in a state of oxidation above the amine. With the para-compound 94% of the amount recovered was excreted during the first 24 hours; 80% was excreted within the first 8 hours. Of the total amount of the para-isomer recovered 2.5% to 4% was free amine, 11% to 20% was conjugated amine, and 76% to 87% was excreted in a state of oxidation above the amine.

The excretion by the rats of 76% to 87% of the p-nitrobenzoic acid in a state of oxidation above the amine is in decided contrast to our results in similar tests with p-nitrobenzenesulfonamide. Only

TABLE II.  
Reduction of Isomeric Nitrobenzoic Acids to Diazotizable Compounds by Rats.

Isomer	Dose,* mg	Route	% of dose recovered	Free amine, mg	Total amine after hydrolysis, mg	Total amine after reduction, mg	Distribution of compounds recovered		
							Free amine, %	Conju- gated amine, %	Not re- duced to amine in animal body, %
Ortho	20	Intra- peritoneal	72.1†	2.45		11.84†	20.6		
Meta	20	"	62.6	0.83	3.07	10.27	8.1	21.8	70.1
Para	20	"	89.4	0.58	3.51	14.67	3.9	20.0	76.1
"	20	Oral	93.4	0.39	2.06	15.33	2.5	10.9	86.6
"	20	"	94.0	0.39	3.52	15.43	2.5	20.3	77.2

\*20 mg nitrobenzoic acid is equivalent to 16.41 mg of aminobenzoic acid.

†The formation of the undiazotizable anhydride, anthranil, during the heating of o-amino-benzoic acid at an acid pH makes these values too low.



5% to 16% of the p-nitrobenzenesulfonamide is excreted in a state of oxidation above the amine.<sup>8</sup>

*Conclusions.* p-Nitrobenzoic and m-nitrobenzoic acids are readily reduced to amino-compounds by tissue suspensions. o-Nitrobenzoic acid, in contrast, is reduced to only a small extent by tissue suspensions.

When p-nitrobenzoic acid or m-nitrobenzoic acid is administered to rats only a small fraction of the dose is excreted in the urine in the reduced form, the remainder is present in a state of oxidation above the amine. The reduced fraction is conjugated to a high degree. o-Nitrobenzoic acid is also reduced by rats; an appreciable amount of free amine is recoverable from the urine.

### 13178

#### Occurrence of Hydrogenase in Nitrogen-Fixing Organisms.\*

A. S. PHELPS AND P. W. WILSON. (Introduced by E. G. Hastings.)

*From the Department of Agricultural Bacteriology, University of Wisconsin, Madison.*

Several years ago this laboratory<sup>1</sup> demonstrated that molecular H<sub>2</sub> specifically inhibits the assimilation of atmospheric N<sub>2</sub> by red clover plants inoculated with *Rhizobium trifolii*. In an effort to determine the mechanism of this unusual type of inhibition, cultures of the bacteria were tested for *hydrogenase*, the enzyme which Stephenson and associates<sup>2</sup> found in several species of heterotrophic organisms catalyzing the reversible reaction:  $H_2 \rightleftharpoons 2H$ . Attempts to find the enzyme in cultures of *Rh. trifolii* failed. Recently Wyss and Wilson<sup>3</sup> showed that H<sub>2</sub> likewise inhibits nitrogen fixation by the free-living *Azotobacter*. Accordingly, species of this organism were tested for *hydrogenase*, and in contrast with the earlier results using the root nodule bacteria the azotobacter species were found to possess an active enzyme. This report presents the evidence proving the existence of the enzyme; a future paper will discuss its properties and possible significance for the mechanism of biological nitrogen fixation.

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\* This work was aided by a grant from the Rockefeller Foundation.

<sup>1</sup> Wilson, P. W., *Biochemistry of Symbiotic Nitrogen Fixation*, Madison, Wis., 1940.

<sup>2</sup> Stephenson, M. S., *Bacterial Metabolism*, London, 1939.

<sup>3</sup> Wyss, O., and Wilson, P. W., *Nat. Acad. Sci., Proc.*, 1941, **27**, 162.

*Methods.* A modification of the Thunberg technic described by Tam and Wilson<sup>4</sup> in which the rate of methylene blue reduction is followed with an Evelyn photometer was used. Changes in the procedure included: solution volume increased to 10 ml; methylene blue concentration decreased to  $10^{-5}$  M; cell concentration reduced so that decolorization time ranged from 30-60 minutes; 620 m $\mu$  filter used instead of 540. The tubes were twice evacuated and 0.95 atmosphere replaced with known gas mixtures bubbled through alkaline pyrogallol. All tests were made at 30°C and at pH 6.5. *Azotobacter* cells were grown on Burk's medium plus 2% agar; cultures of rhizobia were grown on Allison's salts plus 1% sucrose and biotin concentrate.

If the log of the percent oxidized methylene blue is plotted against time, an initial short period of lag is frequently observed, followed by a much longer period during which reduction is linear. The specific rate constant,  $k$ , is equal to 2.303 times the slope of the straight line. This rate constant is a much better measure of the true velocity of reduction than is the usual "time of reduction".

*Methylene Blue Reduction Studies.* Initial proof for the existence of the enzyme was furnished by the data summarized in Table I. Each tube contained only cells, buffer and the indicated atmosphere.

The values for the tubes in the vacuum represent the endogenous rate of reduction, *i. e.*, reduction in the absence of the specific substrate,  $H_2$ , and probably arises from dehydrogenase activity. The observed values of  $k$  show that when air is replaced with He or A, reduction of methylene blue proceeds at about the same rate as the endogenous (vacuum), whereas replacement with  $H_2$  causes a marked acceleration in the rate of reduction. Boiling the cells eliminated the reduction.

*Hydrogen Uptake Experiments.* Preliminary investigations were made of the activity of the enzyme by means of gas uptake experiments in the Warburg apparatus at 34°C. Side-arm flasks of 15 ml volume with provision for aeration were used; approximately one

TABLE I.

Atmosphere in Thunberg tube	$k$ value for methylene blue reduction		
	<i>Az. agile</i>	<i>Az. vinelandii</i>	
Vacuum	.0132	.0174	.033
Helium	.0160	.0067	.037
Argon	.0094	—	—
Hydrogen	.0824	.1196	.212
Hydrogen (boiled cells)	.0006	.0008	—

<sup>4</sup> Tam, R. K., and Wilson, P. W., *J. Bact.*, 1941, **41**, 529.

TABLE II.

Acceptor	$Q_{H_2} (N)$	
	Before addition	After addition
M.B.	511	733
M.B.	503	652
Fumarate	578	822
Fumarate	413	800
$Ca(NO_3)_2$	503	606
$Ca(NO_3)_2$	450	1050
Pyruvate	750	750
Butyraldehyde	559	125
Glucose	600	377

liter of gas of the desired composition was led through each flask to displace the air. The studies showed that even without addition of specific acceptors, uptake of hydrogen was readily demonstrable, probably because of endogenous acceptors in the cells. In order to prove that a given compound could serve as an acceptor, the  $Q_{H_2}(N)$  was determined first on a suspension, then on the suspension plus the compound which had been added from the side-arm. Increase in the  $Q_{H_2}(N)$  following addition was taken as evidence that the compound would unite with  $H_2$  in the presence of the enzyme. Typical results given in Table II show that methylene blue, fumarate, and nitrate serve as hydrogen acceptors but not pyruvate, butyraldehyde, or glucose. Each value for methylene blue, fumarate, and nitrate is from a separate experiment. Endogenous uptake of gas in a helium atmosphere was zero.

Some evidence was secured that  $O_2$  might act as an acceptor for  $H_2$  in experiments with resting cells of *Az. vinelandii* on glucose. The  $Q_{O_2}(N)$  under an atmosphere of 80%  $He$ -20%  $O_2$  was compared with that under an atmosphere of 80%  $H_2$ -20%  $O_2$ ; if the cells assimilated  $H_2$ , the rate of uptake of gas in the  $H_2$ - $O_2$  atmosphere should be greater than in the  $He$ - $O_2$ . In one experiment the  $Q_{O_2}(N)$  for cells in the presence of  $He$ - $O_2$  was 2590 whereas in the presence of  $H_2$ - $O_2$  a value of 4040 was observed. Additional positive experiments, while not so striking, showed definitely greater rates in the  $H_2$ - $O_2$  atmosphere, but in others no significant differences were noted.

*Miscellaneous Observations.* The effect of cyanide and urethane on the activity of the enzyme in *Az. vinelandii* is illustrated in Table III.

These responses are typical of dehydrogenases in general and of hydrogenases reported for other bacteria.

The effect of environment on the occurrence of the enzyme was

TABLE III.

KCN	k value	Urethane	k value
Endogenous	.0053	Endogenous	.0465
Hydrogen	.0639	Hydrogen	.1610
" + M/200 KCN	.0641	" + M/80	.1207
" + M/100	.0685	" + M/8	.0940
" + M/75	.0720		
" + M/50	.0694		
" + M/20	.0083		

determined by cultivating *Az. vinelandii* on a nitrogen-free agar medium and on the same medium plus 200 mg/100 ml of  $\text{NH}_4\text{NO}_3$ . Flasks of both series were filled with the following atmospheres by the method of Wyss and Wilson<sup>3</sup>: (1) air; (2)  $\text{pO}_2$ , 0.2,  $\text{pN}_2$ , 0.2,  $\text{pH}_2$ , 0.6 atm.; (3)  $\text{pO}_2$ , 0.2,  $\text{pN}_2$ , 0.3,  $\text{pH}_2$ , 0.5 atm. Values of  $k$  for the reduction of methylene blue in an atmosphere of  $\text{H}_2$  were then determined for the six suspensions prepared from these treatments after adjusting each to a standard reduction time. Results from this preliminary study indicated that the enzyme is produced by *azotobacter* cells grown under all the conditions used.

A reinvestigation of the occurrence of *hydrogenase* in the rhizobia resulted in a quite unexpected observation. In agreement with our earlier findings, cultures of *Rh. leguminosarum* 311 grown on laboratory media do not possess the enzyme but suspensions taken by the method of Thorne and Burris<sup>5</sup> directly from the nodules of peas inoculated with this strain of rhizobia actively reduce methylene blue in the presence of  $\text{H}_2$ . Preliminary trials with the soybean organism, however, indicate that neither laboratory nor nodular cultures possess the enzyme.

*Summary.* The existence of *hydrogenase*, the enzyme activating molecular  $\text{H}_2$ , has been demonstrated in cultures of *Azotobacter* by a methylene blue reduction method as well as by measurements of  $\text{H}_2$  uptake in the Warburg microrespirometer. The enzyme is found in cells grown in the presence or absence of  $\text{H}_2$  and using either free or combined nitrogen. Cells of the pea organism taken directly from the nodule possess the enzyme but those grown on the usual laboratory media do not.

<sup>5</sup> Thorne, D. W., and Burris, R. H., *J. Bact.*, 1940, **39**, 187.

## 13179 P

**Increased Plasma Prothrombin Activity after Epinephrine Injections; Relation to Hyperglycemia.**

LEANDRO M. TOCANTINS AND JAMES F. O'NEILL.

*From the Division of Hematology and Surgical Service B, Jefferson Medical College and Hospital, Philadelphia.*

Hypercoagulability of the blood often accompanies the hyperglycemia produced in dogs by epinephrine.<sup>1</sup> Similar changes observed in cats after acute hemorrhage, rage and pain were attributed by Cannon and coworkers<sup>2, 3, 4</sup> to the action of epinephrine on the liver. The evidence here presented in preliminary form, indicates that the hypercoagulability brought about by epinephrine is the result, in part at least, of an increase in the prothrombin activity of the plasma.

Prothrombin activity was measured by the method of Quick<sup>5</sup> with minor modifications<sup>6</sup> and the following precautions: (a) All thromboplastin solutions used were potent enough to produce coagulation of undiluted recalcified dog plasma in 8-10 seconds, and of human plasma in 15 to 18 seconds. (b) Dilutions (1-2, 1-4) of each plasma were made and their times compared with that of undiluted or similarly diluted control plasma. (c) Heparin (4 Toronto units) was added to 1 cc of each of the plasmas and the respective prothrombin times compared. (d) Determinations were carried out simultaneously with solutions of thromboplastin of slightly different potency. The percent cell volume of the blood was measured in each specimen. Variations were generally slight. In most instances the coagulation time of venous blood was also measured.

1. Epinephrine and the prothrombin activity of dog plasma (12 experiments on 4 dogs).

Dog S-10. Weight 15.7 kg, fasting; 0.055 mg of epinephrine intravenously over a 2 min. interval. (See Table I.)

2. Epinephrine and the prothrombin activity of normal human plasma (7 experiments on 6 normal men): (See Table II.)

Intramuscular injections of the drug (0.01 mg-0.005 mg per kilo

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<sup>1</sup> Vosburgh, C. H., and Richards, A. N., *Am. J. Physiol.*, 1903, **9**, 35.

<sup>2</sup> Cannon, W. B., and Gray, H., *Am. J. Physiol.*, 1914, **34**, 232.

<sup>3</sup> Cannon, W. B., and Mendenhall, W. L., *Am. J. Physiol.*, 1914, **34**, 251.

<sup>4</sup> Gray, H., and Lunt, L. K., *Am. J. Physiol.*, 1914, **34**, 332.

<sup>5</sup> Quick, A. J., *Am. J. Physiol.*, 1937, **118**, 260.

<sup>6</sup> Hause, W. A., and Tocantins, L. M., *Am. J. Clin. Path.*, 1941, **11**, 54.



TABLE I.

		Time after epinephrine					
		Before	9'	19'	79'	150'	281'
April 16, 1941							
Blood coagulation time (min)*	10½		8	5	5	3	4½
Plasma Proth. (sec)	9.2		8.6	8.2	7.9	8.6	8.7
Plasma + 4 units of heparin (sec)	16.6		15.3	14.9	lost	15.5	15.5

\*1 cc of venous blood at 38°C in paraffined tubes, 17 mm diameter.

TABLE II.

		Epinephrine 0.0025 mg/kg body wt intraven. (2' for injection)					
		Control	Before	27'	60'		
Apr. 14, 1941							
T.	Bl. Coag. time (min)*	29	22	10	15		
	Plasma Proth. time (sec)	16.5	16.5	15.2	16.5		
		Control	Before	22'	80'	135'	195'
J.N.	Bl. Coag. time (min)	27	32	20	21	19	6½
	Plasma Proth. time (sec)	15	15.3	14	14.5	14.4	14

\*1 cc of venous blood at 38°C in paraffined tubes, 17 mm diameter.

body weight) followed by observations at 30, 60 and 120 minutes gave only slight and inconstant results in 3 normal men. Judging from the doses employed by Cannon and Gray<sup>2</sup> in cats our intramuscular doses were probably too small.

3. Epinephrine and the prothrombin activity of abnormal human plasma:

(a) In patients with various types of hepatic disease (11 experiments in 10 patients). In 6 instances there was an increase in prothrombin activity within 1 hour after injection (0.0025 mg epinephrine per kilo body weight, intravenously over a 2-minute period), in 4 there was no change and in 1 there was a slight decrease.

April 7, 1941. B.C., fem., 29. Widespread metastatic carcinoma of the liver. For 2 days before experiment patient received 2 mg of 2-methyl-1,4-naphthoquinone by mouth.

TABLE III.

	Before Epinephrine	Min after injection				Control
		20	60	150	300	
Prothrombin time (sec)	20.6	18.9	19.9	20.2	19	17.5
% prothrombin	55	80	67	65	78	100

(b) In patients with hemophilia (5 experiments, 4 patients). In 3 instances a significant increase in prothrombin activity was observed, in 2 the change was insignificant. There were no significant changes in the coagulability of whole blood.

S.M., male, age 23. Epinephrine (0.0025 mg per kg body weight) injected intravenously over a 2-minute period.

TABLE IV.

	Before	Min after injection				Control
		5	60	184	274	
Prothrombin time (sec.)	17.8	15.3	16.8	16	17.9	18
% prothrombin	110	400	155	250	110	100
Blood coagul. time (min)	84	75	74	74	54	

*Comment.* Current work on variations in prothrombin has dealt mostly with diminutions in this substance. Quantitative fluctuations in prothrombin must, however, be found obviously in either direction from normal values, as with other components of the plasma. Observations to be reported elsewhere point to the occurrence of pronounced increases in prothrombin activity of the plasma after a meal especially rich in protein, after emotional excitement and during parturition. In all of these states variable degrees of hyperglycemia may be found. Attention has been drawn<sup>7</sup> to an as yet unexplained relationship between sugar metabolism and coagulation of the blood. Removal of liver tissue is followed by a rapid decrease in blood prothrombin<sup>8</sup> and, likewise, by a profound hypoglycemia.<sup>9</sup> The common nature of the forces that lead to hyperglycemia and increased coagulability of the blood has led Stuber and Lang<sup>7</sup> to attribute to glucose an important rôle in blood coagulation. Perhaps a more likely interpretation is that both glycogenolysis and prothrombin production or liberation into the blood are functions of the liver affected in a common manner by identical forces.

*Summary.* Epinephrine, by slow intravenous injection, increases the prothrombin activity of human and dog plasma. An identity of type seems to exist between the forces capable of exciting hyperglycemia and increases in plasma prothrombin activity.

<sup>7</sup> Stuber, B., and Lang, K., *Physiologie und Pathol. der Blutgerinnung*, Berlin und Vienna, 1930.

<sup>8</sup> Warner, E. D., *J. Exp. Med.*, 1938, **68**, 831.

<sup>9</sup> Mann, F. C., and Magath, T. B., *Arch. Int. Med.*, 1922, **30**, 73.

## Mode of Action of Gramicidin.

DOROTHY HEILMAN AND WALLACE E. HERRELL. (Introduced by J. L. Bollman.)

*From the Division of Experimental Medicine, Mayo Foundation, and the Division of Medicine, Mayo Clinic, Rochester, Minn.*

Gramicidin has been found to be bactericidal in small amounts for Gram-positive bacteria growing in tissue culture media.<sup>1</sup> The relative sensitivity of the bacterial species studied coincides with the results of bactericidal tests reported by Dubos.<sup>2</sup> Gramicidin is also hemolytic in bactericidal concentrations;<sup>3, 4</sup> however, similar amounts of the substance do not appear to be toxic for granulocytes, fibroblasts or cells of the lymphoid series growing in tissue culture.<sup>5</sup> These qualities, (1) bactericidal activity, (2) hemolytic activity and (3) low toxicity for tissues, are also characteristic of some substances generally classified as detergents. Moreover, gramicidin shows a definite specificity of bactericidal action. The growth of pneumococci and hemolytic streptococci is inhibited by small amounts of the substance. Larger amounts are necessary to inhibit other kinds of streptococci and relatively large amounts must be used to inhibit staphylococci. Gram-negative bacteria in general are not affected. A similar type of bactericidal selectivity has been shown for soaps of the unsaturated fatty acids by Lamar,<sup>6</sup> Avery,<sup>7</sup> Walker,<sup>8</sup> Eggerth,<sup>9</sup> and Bayliss and Halvorson,<sup>10</sup> and for sodium lauryl sulfate by Birkeland and Steinhaus.<sup>11</sup> Baker, Harrison and Miller<sup>12</sup> recently showed that anionic synthetic detergents display the same kind of specificity in their inhibitory effect on the metabolism of various bacterial species.

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<sup>1</sup> Herrell, W. E., and Heilman, Dorothy, *J. Clin. Invest.*, in press.

<sup>2</sup> Dubos, R. J., *J. Exp. Med.*, 1939, **70**, 1.

<sup>3</sup> Dubos, R. J., *Ann. Int. Med.*, 1940, **13**, 2025.

<sup>4</sup> Heilman, Dorothy, and Herrell, W. E., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 182.

<sup>5</sup> Herrell, W. E., and Heilman, Dorothy, unpublished data.

<sup>6</sup> Lamar, R. V., *J. Exp. Med.*, 1911, **13**, 380.

<sup>7</sup> Avery, O. T., *J. A. M. A.*, 1917, **71**, 2050.

<sup>8</sup> Walker, J. E., *J. Infect. Dis.*, 1924, **35**, 557.

<sup>9</sup> Eggerth, A. H., *J. Exp. Med.*, 1929, **50**, 299.

<sup>10</sup> Bayliss, Milward, and Halvorson, H. O., *J. Bact.*, 1935, **29**, 9.

<sup>11</sup> Birkeland, J. M., and Steinhaus, E. A., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 86.

<sup>12</sup> Baker, Zelma, Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **72**, 249.

As a rule the hemolytic activity of a detergent coincides with its bactericidal activity. Lamar found that soaps which were most bactericidal against the pneumococcus were also most active in causing hemolysis. Eggerth,<sup>13</sup> in a study of alpha-brom soaps of saturated fatty acids, concluded that the bactericidal activity paralleled the hemolytic activity. Katz and Lipsitz,<sup>14</sup> reporting on a synthetic detergent derived from naphthalene sulfonic acid, stated that a concentration of this substance which would inhibit the growth of *Mycobacterium smegmatis* also caused hemolysis.

From cultures of the soil bacillus which yields gramicidin, Hotchkiss and Dubos<sup>15</sup> have isolated a related substance, tyrocidine, which is less bactericidal for Gram-positive organisms than gramicidin, but which is effective against Gram-negative as well as Gram-positive bacteria. It has been pointed out previously that tyrocidine resembles the cationic detergents in its bactericidal activity. The cationic detergents have been shown by Baker, Harrison and Miller to inhibit the metabolism of Gram-positive and Gram-negative organisms to the same degree.

In view of some points of similarity between gramicidin and tyrocidine and certain detergents a study was made of the ability of gramicidin and tyrocidine to depress the surface tension of aqueous solutions. The samples of tyrocidine and gramicidin used in this study were prepared by Osterberg from tyrothricin, the crude bactericidal substance, by the method of Hotchkiss and Dubos. The tyrothricin was supplied by Sharp and Dohme. Tyrocidine is partially soluble in water; however, gramicidin is quite insoluble in this medium. As a result, 2% solutions of these substances in 95% alcohol were added to the solutions to be tested. The resulting suspensions and solutions used as controls contained 0.475% of alcohol. The surface tensions of these suspensions were determined with a precision type DuNoüy tensiometer within an hour of their preparation. It was observed that suspensions of gramicidin showed a rapid decrease of surface tension within the first 10 minutes after being placed in the watch glass for study with the tensiometer; hence readings were made at 3 intervals, the first immediately after the material was placed in the watch glass, and the second and third at 10 and 30 minutes thereafter. This initial drop of surface tension with time occurred regardless of how long the suspension had been prepared before the observations on surface tension were made.

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<sup>13</sup> Eggerth, A. H., *J. Exp. Med.*, 1929, **49**, 53.

<sup>14</sup> Katz, Joseph, and Lipsitz, Aaron, *J. Bact.*, 1935, **30**, 419.

<sup>15</sup> Hotchkiss, R. D., and Dubos, R. J., *J. Biol. Chem.*, 1940, **136**, 803.

The rapid decrease of surface tension with time has been described by DuNoüy<sup>16</sup> in the case of certain colloidal solutions. The solutions prepared with 0.9% solution of sodium chloride were adjusted to pH 7.2 and the preparations made with Tyrode's solution had a pH between 8.0 and 8.2. Similar amounts of sodium oleate and a synthetic detergent, dioctyl ester of sulfonated succinic acid (aerosol OT) were included in some of the tests for comparison. The results of these tests are shown in Fig. 1.

It may be seen that tyrocidine was effective in lowering the surface tension regardless of the kind of solution used. Gramicidin is less active in water or saline solutions but when its solubility is in-

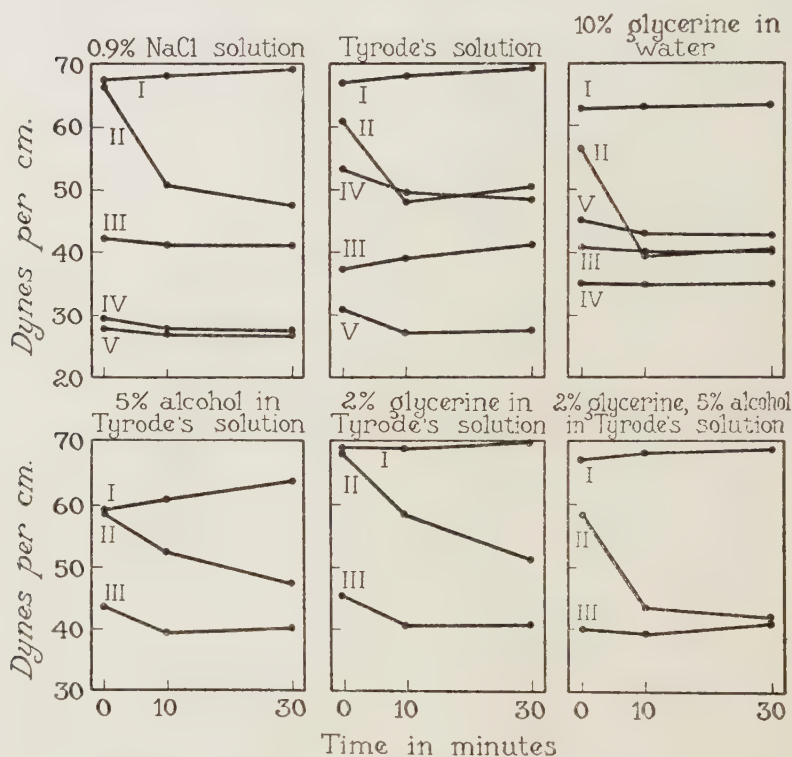


FIG. 1.

Effect of gramicidin, tyrocidine, sodium oleate and aerosol OT on surface tension of various solutions at 25°C. Key to curves (type of solution concerned in each chart is given in its caption) is as follows: I, solution with 0.5% alcohol added; II, solution same as I, gramicidin added 1 to 10,000; III, solution same as I, tyrocidine added 1 to 10,000; IV, solution same as I, sodium oleate added 1 to 10,000; V, solution same as I, aerosol added 1 to 10,000.

<sup>16</sup> Du Noüy, P. L., *Surface Equilibria of Biological and Organic Colloids*, New York, The Chemical Catalog Company, 1926, 212 pp.



creased by the addition of 10% of glycerin or 2% glycerin and 5% alcohol, its ability to lower the surface tension after 30 minutes of observation approaches that of tyrocidine. It may be seen that sodium oleate is less active in Tyrode's solution, presumably because of the presence of calcium and magnesium ions.

A study was made of the effect of pH on the activity of tyrocidine and gramicidin. Concentrations of 1:5000 of these substances were prepared in fifteenth-molar phosphate buffers (Sörensen). There was no significant change in the activity of either substance over a pH range of 5.3 to 8.0. Likewise, heating suspensions of gramicidin in water or Tyrode's solution to 90°C for 10 minutes did not appear to inhibit their surface tension depressing ability when these suspensions subsequently were added to 2% glycerin in water or 10% rabbit's serum in 0.9% solution of sodium chloride.

The inhibitory effect of serum on the bactericidal action of soaps has been pointed out by Eggerth,<sup>17</sup> Walker,<sup>8</sup> and others. The antagonistic effect of serum on the surface tension depressing activity of sodium oleate is discussed by DuNoüy.<sup>15</sup> Table I records the effect of increasing amounts of rabbit's serum in inhibiting the surface tension depressing activity of gramicidin, tyrocidine, tyrothricin (the crude bactericidal substance containing about 85% of tyrocidine and 15% of gramicidin), sodium oleate and aerosol OT. Tyrocidine shows a remarkable degree of activity in the presence of large amounts of serum. The crude bactericidal substance appears to be somewhat

TABLE I.  
Effect of Rabbit's Serum on Depression of Surface Tension of Saline Solutions by 1:5,000 Concentrations of Gramicidin, Tyrocidine, Tyrothricin, Sodium Oleate and Aerosol OT.

	Gramicidin	Tyrocidine	Tyrothricin	Sodium oleate	Aerosol OT
0.9% NaCl solution	18*	26	26	40	41
1% serum in .9% NaCl sol.	14	20	21	31	31
5% serum in same	10	19	18	18	24
10% " " "	8	17	17	10	17
20% " " "	4	14	16	4	10
40% " " "	3	18	17	1	6
Tyrode's solution	20	28	27	19	40
1% serum in Tyrode's sol.	12	19	21	13	32
5% serum in same	6	17	19	9	24
10% " " "	4	15	16	8	16
20% " " "	4	15	15	4	10

\*Values indicate the difference in dynes between the surface tension of the original solution and that of the same solution to which the various substances have been added. Determinations were made at 10 minutes. Temperature 25°C. All solutions were adjusted to pH 7.6.

<sup>17</sup> Eggerth, A. H., *J. Exp. Med.*, 1927, **46**, 671.

more active than would be expected from its content of gramicidin and tyrocidine. The inhibitory effect of increasing amounts of serum appears to be proportionately about the same for gramicidin and aerosol OT, but it is much greater in the case of sodium oleate, either in the presence of 0.9% solution of sodium chloride or in Tyrode's solution.

It is evident that the relative ability of gramicidin and tyrocidine to depress the air-liquid interfacial tension of the solutions studied is not proportional to their respective bactericidal activities against Gram-positive bacteria or to their hemolytic activity. The effectiveness of gramicidin appears to depend on its partial solubility. It is possible that the activity of gramicidin might be altered by traces of organic solvents or by enzyme action at the cell surface. However, the fact that tyrocidine is more active in depressing surface tension under all conditions studied might indicate that the greater bactericidal action of gramicidin on Gram-positive organisms depends on a more specific activity of this substance. Such a possibility seems more likely in view of the fact that heating destroys the bactericidal and hemolytic properties of gramicidin but does not alter its ability to depress surface tension. It would seem that the tendency of this substance to concentrate at an interface might enhance any such specific metabolic activity greatly.

*Conclusions.* 1. Gramicidin and tyrocidine depress the surface tension of aqueous solutions. Tyrocidine is more active in this regard than is gramicidin. 2. The ability of gramicidin to depress surface tension is improved by the addition of organic solvents such as glycerin which increases the solubility of gramicidin. 3. Serum decreases the activity of tyrocidine less than it does the activity of gramicidin, sodium oleate and aerosol OT. 4. The bactericidal and hemolytic effects of gramicidin are destroyed by heat but its property of altering surface tension is heat stable.

## 13181 P

## Parasiticial Properties of the Proteolytic Enzyme Ficin.\*

E. C. FAUST AND L. F. THOMEN.

From the Department of Tropical Medicine, Tulane University of Louisiana,  
New Orleans, La.

For centuries natives of Tropical America have used the crude fresh latex of *Ficus glabrata* and *F. doliaria* as an anthelmintic, especially for the removal of the human whipworm (*Trichocephalus trichiurus*).<sup>1</sup> However, unless refrigerated or chemically preserved, the latex ferments rapidly. Robbins<sup>2</sup> isolated the anthelmintic principle *ficin* in amorphous form, while more recently Bliss<sup>3</sup> has prepared a semi-refined crystoid and Walti<sup>4</sup> a pure crystal product.

The present study was undertaken to compare the parasiticial properties of the refrigerated and chemically preserved (0.1-1.0% sodium benzoate) latex, the semi-refined crystoid (sealed *in vacuo*) and the amorphous ficin.<sup>†</sup>

*In vitro* tests were made on egg albumin coagulated by heat in capillary tubes 25 mm long by 2.5 mm inside diameter, on living pig *Ascaris* and on living dog whipworms. The products tested *in vitro* were the undiluted refrigerated and preserved crude latex, 1, 2, 3, 4, 5, and 10% dilutions of these products in 0.85% sodium chloride, and 3 and 10% dilutions in artificial gastric juice; likewise 0.01, 0.1, 1, 5, and 10% solutions of the crystoid in distilled water, 0.85% sodium chloride and artificial gastric juice. The test objects were immersed in the samples to be tested, were incubated at 36-37°C, and were examined at regular intervals up to 24 hours. The amount of digestion of egg albumin from the ends of the tubes provided an index of proteolytic efficiency of the product, while the lessened activity of the worm and state of destruction of its cuticle served as criteria of anthelmintic potency. Controls in physiological salt solution were set up in each experiment.

*In vivo* observations were made on 80 dogs, infected with hook-

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\* Aided by a grant from Merck and Company, Rahway, N. J.

1 Thomen, L. F., *Am. J. Trop. Med.*, 1939, **19**, 409.

2 Robbins, B. H., *J. Biol. Chem.*, 1930, **87**, 251.

3 Bliss, S. W., personal communication.

4 Walti, A., *J. Am. Chem. Soc.*, 1938, **60**, 493.

† With the exception of one sample of refrigerated latex supplied by Dr. H. C. Clark, Director of the Gorgas Memorial Laboratory, Panama, R. de P., and preserved latex obtained from Higueronia Laboratories, Mexico, D. F., all of the products tested have been obtained from Merck and Company.

worms, whipworms, ascarids, tapeworms, and *Endamoeba histolytica*. Tests were conducted with refrigerated and preserved latex, the cystoid, and the amorphous ficin. The study included pre-treatment and post-treatment Stoll egg counts of hookworms and whipworms. Measured amounts of the drugs were administered on the basis of body weight. Tests were conducted with and without pre-treatment purgation or enemata. After therapy and follow-up studies, each animal was anesthetized and the entire digestive tract examined to determine the results of the treatment.

All of the *in vitro* tests demonstrated some degree of digestive action upon the test objects. The refrigerated unpreserved samples of latex were most active, digesting one mm length of coagulated albumin in 60 minutes, digesting the cuticle of *Ascaris* within 30 minutes in a dilution up to 1:10 in 0.85% sodium chloride and disintegrating the whipworms within 2 hours in a 1:20 dilution. The Higueronia product was about 25% less efficient. A 10% solution of the freshly opened cystoid in 0.85% sodium chloride was comparable to the undiluted or 10% sample of refrigerated latex. In all cases 0.85% sodium chloride used as a diluent was found to provide a higher potency than distilled water, while gastric juice appeared partially to inactivate the enzyme.

The *in vivo* experiments provided certain conclusive results. Administration of one ounce of the refrigerated latex to each of 5 unpurged dogs removed all of the whipworms. Similar results were obtained with preserved latex (5 animals) following pre-treatment purgation. With the freshly opened cystoid single doses of 0.6 to 3 g per kilo of body weight (9 animals) or equivalent amounts in 2 divided doses (6 animals) or 4 divided doses (2 animals) were equally effective. Lesser amounts of the cystoid (2 animals) were 25 to 70% efficient. However, when exposed to air for only 2 or 3 days the cystoid (7 animals) rated only 0 to 25%. The amorphous ficin (24 animals), although relatively stable on exposure to air, required a total minimum of one gram per kilo of body weight, in single or divided doses, to produce 100% removal of whipworms, but if the bowel was obstructed by tapeworms or feces, as much as 4 g per kilo were ineffective.

Comparable ratios of efficiency among the several test products were obtained on hookworms but the specificity was invariably less than on whipworms. Intragastric intubation of a solution of the cystoid or amorphous ficin was usually more efficient than oral administration in gelatin capsules. The action of no product was consistently efficient for dog ascarids; the efficiency for tapeworms



was essentially *nil*. In 10 animals treated with the fresh cystoid and 14 with the amorphous ficin, trophozoites of *E. histolytica* in the wall of the large bowel quite uniformly became dwarfed and inactive, and at times had died *in situ*.

*Summary.* The unpreserved, refrigerated crude latex was consistently the most efficient parasiticide tested, the preserved latex somewhat less effective. A 10% solution of the freshly opened cystoid compared favorably with the refrigerated latex but exposure to air rapidly diminished its potency. The amorphous ficin, while relatively stable, proved to be only about half as efficient as the fresh cystoid. The most specific action of these products occurred on whipworms, provided the large bowel was previously free of feces. The effect on hookworms was almost uniformly less satisfactory. Considerable amebostatic and, at times, amebicidal action was demonstrated.

## 13182

## Determination of Ascorbic Acid in Whole Blood.\*

CARL A. KUETHER AND JOSEPH H. ROE.

*From the Department of Biochemistry, School of Medicine, George Washington University.*

It has been found that ascorbic acid is oxidized by the oxygen set free from oxyhemoglobin when whole blood is deproteinized with  $\text{HPO}_3$ , and that this oxidation can be prevented by displacing the oxygen of oxyhemoglobin with  $\text{CO}$ ,  $\text{N}_2$ , and  $\text{H}_2$ .<sup>1-3</sup> Butler and Cushman<sup>4</sup> have reported a method for the determination of ascorbic acid in whole blood involving saturation of the blood with  $\text{CO}$  and precipitation of the proteins under an atmosphere of  $\text{CO}$ .

We have found that a very convenient method to prevent the oxidative effect of oxyhemoglobin is alternate removal of oxygen by evacuation and treatment with  $\text{CO}_2$  under pressure. A combination of these two steps gives an efficient and practical way of reducing oxyhemoglobin which is adaptable to clinical laboratory use.

\* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

1 Lemberg, R., and Legge, J. W., *J. Proc. Roy. Soc. N. S. Wales*, 1938, **72**, 62.

2 Lemberg, R., Cortis-Jones, B., and Norrie, M., *Biochem. J.*, 1938, **32**, 149.

3 Huzita, A., Ebihara, T., and Numata, I., *Biochem. Z.*, 1939, **301**, 245.

4 Butler, A. M., and Cushman, M., *J. Clin. Invest.*, 1940, **19**, 459.



Five cc of blood are pipetted into a cylindrical separatory funnel with a mark at 20 cc, containing 2 drops of octyl alcohol as an antifoam. The glass stopper of the funnel has a removable clamp by means of which it may be held in position. The funnel is connected by means of pressure tubing to the single outlet on one side of a 3-way stopcock. One of the other outlets is connected to a vacuum line through a manometer, and the third outlet is connected to a tank of  $\text{CO}_2$  with a manometer and a gas reservoir in the line to give it a larger volume and thus minimize the pressure changes. We have used a volleyball for the reservoir and found it very satisfactory. The funnel is then evacuated to a pressure of about 5 mm Hg until the evolution of gas stops. We have found that 2 minutes is usually sufficient time to do this. After filling the funnel with  $\text{CO}_2$ , both the stopper and stopcock are opened and  $\text{CO}_2$  run through to wash out any residual air remaining in the funnel. The clamp is then placed on the stopper and  $\text{CO}_2$  run in at a pressure of about 76 cm Hg above atmospheric pressure for 5 minutes. During evacuation and  $\text{CO}_2$ -pressure treatment the funnel is rotated gently and continuously. *It is very important to spread the blood in a thin layer over the inner surface of the funnel during the evacuation and saturation.* The evacuation and saturation are repeated twice. After the third saturation, the funnel is removed from the  $\text{CO}_2$  pressure line. Five cc of distilled water, from which *all  $\text{O}_2$  has been removed* with a stream of  $\text{CO}_2$ , is then introduced into the funnel to lake the blood. The stopper is immediately replaced and the contents of the funnel mixed. Ten cc of 10%  $\text{HPO}_3$ , from which the  $\text{O}_2$  has been removed with  $\text{CO}_2$ , is then introduced, the volume is made up to the 20 cc mark, and the contents of the funnel are thoroughly mixed by shaking and filtered through a dry filter paper.

The filtrate obtained in this manner is analyzed for reduced ascorbic acid by a modification of the method of Mindlin and Butler.<sup>5</sup> The galvanometer of the photoelectric colorimeter (Evelyn) is set to read 100 with a tube of blank dye solution which has been completely decolorized with ascorbic acid. A blank is run by measuring 4 cc of 3%  $\text{HPO}_3$  into a colorimeter tube, adding 4 cc of the indophenol-acetate solution and reading in the colorimeter. The indophenol-acetate solution contains 17 g of  $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$  and approximately 15 mg of indophenol per liter.

Four cc of the filtrate are measured into a colorimeter tube, the tube is inserted into the colorimeter and 4 cc of indophenol-acetate

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<sup>5</sup> Mindlin, R. L., and Butler, A. M., *J. Biol. Chem.*, 1937-38, **122**, 673.

solution are added. The galvanometer is read 15 and 30 seconds after addition of the dye. A few crystals of ascorbic acid are added and the reading of the decolorized solution is taken after 5 minutes.

The final pH of a mixture of equal volumes of the dye solution and filtrate as described above is about 3. At a pH appreciably lower than this, as shown by Bessey,<sup>6</sup> there is decolorization of the dye by hydrogen ion, and at higher pH values the decolorization of the dye by interfering substances (principally sulfhydryl) is appreciable. Bessey<sup>6</sup> carries out this determination at pH 3.5-3.6. Mindlin and Butler<sup>5</sup> use pH 4.1. We have found pH 3 to be optimum for this reaction.

The amount of ascorbic acid in the sample is given by the following equation:

$$\text{Mg Ascorbic Acid} = K[\log (G_{s_{15}} - (G_{s_{30}} - G_{s_{15}})) + (2 - \log G_d) - \log G_b]$$

where K is the constant (0.086, Mindlin and Butler<sup>5</sup>) determined by analyzing standard solutions of ascorbic acid in 3%  $\text{HPO}_3$ ,  $G_{s_{15}}$  and  $G_{s_{30}}$  are the 15 and 30 second readings, respectively,  $G_d$  is the decolorized reading of the sample and  $G_b$  is the reading of the blank solution. The first term of the equation includes a correction for the drift in the galvanometer reading produced by the non-ascorbic acid reducing substances released when the erythrocytes are hemolyzed. The second term is a correction for any turbidity which the solution may have. We have found that filtrates which to the eye appear to be perfectly clear will not give a reading of 100 when decolorized, and therefore this correction is necessary.

In 9 analyses of normal human blood, and normal and scorbutic guinea pig blood to which pure ascorbic acid was added, the following recoveries of the added ascorbic acid were obtained: 90, 92, 98, 99, 102, 102, 103, 108 and 110%.

*Summary.* A method for the determination of ascorbic acid in whole blood is proposed. The oxidation of ascorbic acid by oxyhemoglobin is prevented by reduction of the oxyhemoglobin by alternate evacuation and treatment with  $\text{CO}_2$  under pressure before deproteinization with  $\text{HPO}_3$ .

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<sup>6</sup> Bessey, O. A., *ibid.*, 1938, **126**, 771.

## Effects of Clinical Doses of Phenobarbital on Blood and Urine Ascorbic Acid in Human Subjects.

SYDNEY T. WRIGHT, TOM J. CALLAGHAN, AND VIRGINIA MINNICH.  
(Introduced by W. H. Olmsted.)

*From the Department of Medicine, Washington University, St. Louis, Mo.*

That barbituric acid derivatives were effective in stimulating ascorbic acid excretion in rats was demonstrated by Longenecker and his co-workers.<sup>1</sup> Within 5 days the administration of sodium phenobarbital caused an increase of 15 times in the urinary excretion of ascorbic acid.

This experiment records the effects of clinical doses of phenobarbital on the plasma, whole blood, and urinary values of ascorbic acid in human subjects. Although some other drugs are equally effective in stimulating ascorbic acid excretion in animals, phenobarbital was chosen because it is so commonly used in clinical medicine. It should be recalled that a daily dose of 20 mg of sodium phenobarbital was given by King and his co-workers to a 250 g rat, whereas we administered 180 mg to a 70K subject.

In rats, it was shown that 5 days were required to stimulate the maximum excretion of ascorbic acid, so a 5-day period of phenobarbital administration was chosen for the human subjects. The 180 mg daily dose did affect the subjects in so much that dizziness and diplopia were notable on the 5th day.

*Plan of Experiment.* Three subjects were used. Subject A was a well developed male weighing 170 pounds, subject B a well developed male weighing 185 pounds, and subject C a normal adult female weighing 125 pounds. The following diet was consumed each day during the entire experiment:

Food	g	Food	g	Food	g
Am. cheese	20	Figs, dried	30	Pork	75
Bacon	10	Flour	3	Puffed wheat	15
Beef	75	Grape jelly	30	Ripe olives	30
Butter	45	Grape juice	100	Soda crackers	12
Cream	170	Jello	16	Sugar	13
Dates	20	Limas, dried	20	Walnuts	15
Eggs	2	Macaroni	10	W. W. bread	120
Evap. milk	200	Peas, dried	20	Total calories	3021

Total calories in individual diets were adjusted by varying the amount of bread, milk, cream, butter, and flour according to the

<sup>1</sup> Longenecker, H. E., Fricke, H. H., and King, C. G., *J. Biol. Chem.*, 1940, **135**, 497.

individual preferences of the 3 subjects. During the month of the experiment the weights of the subjects did not change.

The vitamin C free diet was supplemented with 25 mg of pure ascorbic acid administered with the morning meal. The constancy of the diet ruled out variations in ascorbic acid excretion due to changes in pH of urine. Ascorbic acid and phenobarbital were taken at approximately the same time each day. It was hoped to arrange the ascorbic acid intake so that blood, and urinary changes would be evident. The subjects voided frequently enough so that urine remained in the bladders of the subjects for only a short period of time.<sup>2</sup> The urine volumes were quite constant, that of subjects A and B averaged 1250 cc daily, while that of subject C averaged 889 cc daily. The subjects performed the same work daily either in the hospital or laboratory. Following a 10-day control period, phenobarbital was taken for a period of 5 days. A second control period followed the drug period.

*Chemical Methods.* Fasting daily specimens of blood were taken. The plasma and whole blood methods of Mindlin and Butler<sup>3</sup> and Butler and Cushman<sup>4</sup> were used to determine the ascorbic acid values.

The urine ascorbic acid was determined on 24 hour specimens by the method of Roe and Hall<sup>5</sup> adapted to the Evelyn photoelectric colorimeter. Recovery experiments on this method gave 90% to 99% recovery of added ascorbic acid.

Results are recorded in Table I.

It is evident that there was a very slight but definitely steady decline in whole blood, plasma, and urinary excretion of ascorbic acid during the 3 weeks of observation in all 3 subjects. In both male subjects, no significant change took place in the ascorbic acid metabolism during the administration of phenobarbital. In the one female subject, there appeared a slight but significant rise in ascorbic acid excretion and decrease in the whole blood values during administration of phenobarbital. We cannot explain this observation.

If ascorbic acid were used by the human being in detoxifying phenobarbital as Longenecker, *et al.*, believe occurs in the rat, we would expect a decrease in the blood value for ascorbic acid and a decrease in urinary output during phenobarbital administration to man. It is possible that if on the basis of weight the dosage to

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<sup>2</sup> Sherry, S., and Friedman, G. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **42**, 707.

<sup>3</sup> Mindlin, R. L., and Butler, A. M., *J. Biol. Chem.*, 1938, **122**, 673.

<sup>4</sup> Butler, A. M., and Cushman, M., *J. Clin. Invest.*, 1940, **19**, 459.

<sup>5</sup> Roe, J. H., and Hall, J. M., *J. Biol. Chem.*, 1939, **128**, 329.



TABLE I.  
Ascorbic Acid Concentration of Whole Blood, of Plasma and of Urine.

Date 1941	A				B				C			
	Whole blood mg%	Plasma mg%	Urine mg%	Urine 24 hr mg	Whole blood mg%	Plasma mg%	Urine mg%	Urine 24 hr mg	Whole blood mg%	Plasma mg%	Urine mg%	Urine 24 hr mg
2-5	.84	.40	.61	5.20	.63	.22	.38	4.40				
2-6	1.22	.46	.45	5.48	1.12	.31	.32	4.13				
2-7	1.27	.43	.46	4.80	.86	.33	.28	3.47				
2-8	.99	.43	.39	4.84	1.20	.40	.34	2.43				
2-9	1.12	.58	.35	3.94	.89	.55	.20	1.86	1.12	.58	.34	2.90
2-10	.96	.40	.69	7.54	1.07	.34	.58	6.84	1.20	.54	.89	6.30
2-11	1.37	.42	.48	6.73	.63	.39	.31	3.91	1.37	.60	.66	2.15
2-12	1.36	.40	.48	4.90	1.04	.37	.27	4.90	1.25	.55	.43	3.30
2-13	.79	.34	.46	2.59	.71	.36	.44	5.20	1.22	.50	.45	4.50
2-14	.76	.30	.43	4.18	.73	.30	.31	3.85	.92	.51	.40	3.89
2-15	.77	.32	.50	5.76	.64	.21	.35	3.89	1.00	.47	.43	4.65
Phenobarbital gr iii h.s.												
2-16	.56	.32	.19	2.31	.62	.23	.12	2.18	.68	.52	.28	3.43
2-17	.86	.26	.25	3.53	.76	.14	.27	2.50	.95	.39	.47	3.60
2-18	.80	.31	.49	6.32	.66	.19	.42	5.75	.80	.38	.68	5.60
2-19	.63	.16	.41	5.27	.57	.14	.28	5.34	.70	.32	.53	5.68
2-20	.73	.25	.31	4.30	.52	.16	.27	3.70	.56	.35	.56	4.92
Drug discontinued												
2-21	1.10	.24	.24	2.64	.72	.23	.22	2.92	1.03	.33	.38	3.40
2-22	.60	.20	.32	5.51	.65	.20	.25	3.40	.73	.38	.54	3.80
2-23	.64	.27	.25	3.47	.53	.23	.20	5.30	.74	.32	.36	3.97
2-24	.67	.25	.35	3.50	.67	.15	.34	3.59	.81	.32	.52	3.30
2-25	1.05	.20	.33	4.95	1.11	.15	.24	2.40				
2-26	.50	.16	.24	3.66	.46	.16	.24	2.40				
2-27	.64	.16	.13	1.15	.64	.13	.24	2.40				
Avg												
1st control	1.04	.41	.48	5.09	.87	.34	.34	4.08	1.15	.54	.52	3.96
Drug	.72	.26	.33	4.35	.63	.17	.27	3.89	.74	.39	.50	4.65
2nd control	.74	.21	.27	4.55	.68	.18	.25	3.20	.83	.34	.45	3.62

humans had been comparable to that given by Longenecker to rats some effect would have been noted. It is possible the drug was conjugated with ascorbic acid in the urine, but this does not occur in rats and one would expect in this case some reduction in ascorbic acid values in whole blood or plasma.

*Summary.* There was no significant change in whole blood, plasma, or urinary excretion of ascorbic acid following administration of 180 mg of phenobarbital daily to human subjects. Twenty-five mg of crystalline ascorbic acid daily is insufficient to maintain whole blood or plasma values when the subjects take an ascorbic acid-free diet.



## 13184 P

## Adsorbents for Procaine-Epinephrine Solutions.

F. S. WOZNIAK, J. R. RUSSELL AND A. B. LUCKHARDT.

*From the W. G. Zoller Memorial Dental Clinic and Department of Physiology,  
University of Chicago.*

Mixtures of procaine and epinephrine have long been employed as local anesthetics. Epinephrine, by causing local vasoconstriction, holds the procaine near the injected area, localizing and prolonging the anesthesia. There is risk, however, of the epinephrine entering the general circulation, especially when the anesthetic is injected into the vascular tissue of the gums and palate in dental surgery. The undesirable effects of epinephrine in dental anesthetics have been emphasized by Miller,<sup>1</sup> Stuart,<sup>2</sup> and Tainter, Thronrdson and Moose,<sup>3-5</sup> whose clinical studies show various degrees of rise in blood pressure, increase in heart rate, extrasystoles and increase in respiratory rate following the submucosal injection of procaine-epinephrine solutions. Pickering, McCooey, Steinmeyer and Luckhardt<sup>6</sup> injected procaine-epinephrine solutions in dogs in sites commonly used in dental operations and obtained marked rises in blood pressure.

*Methods.* The purpose of the present experiments is to find a substance which will retard and diminish the undesirable pressor action of epinephrine and procaine in the main circulatory system, while still allowing it to prolong the anesthesia by local vasoconstriction. An adsorbent for epinephrine which will produce such a retarding effect is now being sought. It would also be desirable for procaine to be adsorbed and to be given off slowly as needed to maintain anesthesia. Colloidal tri-calcium phosphate, used extensively as an adsorbent for proteins, was the first substance tested.

Dogs anesthetized with paraldehyde were used in these experiments. Both vagi were sectioned to prevent reflex slowing of the heart by the carotid sinus and aortic arch mechanisms. Blood pressure and respiratory rate and amplitude were recorded on kymo-

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1 Miller, H. C., *J. Am. Dental Assn.*, 1937, **24**, 515.

2 Miller, J. C., and Stuart, C. W., *J. A. D. A.*, 1936, **23**, 1883.

3 Tainter, M. L., Thronrdson, A. H., and Moose, S. M., *J. A. D. A.*, 1937, **24**, 376.

4 Tainter, M. L., Thronrdson, A. H., and Moose, S. M., *J. A. D. A.*, 1938, **25**, 1321.

5 Tainter, M. L., and Thronrdson, A. H., *J. A. D. A.*, 1938, **25**, 966.

6 Pickering, P. P., McCooey, C. J., Steinmeyer, H. P., and Luckhardt, A. B., *J. A. D. A.*, 1939, **26**, 1823.

graph paper in the usual manner. As a control, 2 cc of 2% procaine containing 1:25,000 epinephrine was injected directly into the anterior palatine, mental and posterior palatine foramina. The plunger of the syringe was always retracted prior to the injection to exclude the possibility of direct entrance of the solution into a vein. The time of injection was kept as constant as possible and averaged about 20 seconds.

To test the effect of the colloidal  $\text{Ca}_3(\text{PO}_4)_2$  on epinephrine, 2 cc of  $\text{Ca}_3(\text{PO}_4)_2$  containing 1:25,000 epinephrine was injected into the foramina on the side opposite those employed in the control part of the experiment, using the same dogs. The effect of intravenous injection of  $\text{Ca}_3(\text{PO}_4)_2$  with 1:25,000 epinephrine was compared with intravenously injected 2% procaine with 1:25,000 epinephrine. Chemical tests for adsorption were also made. The supernatant fluid from centrifuged  $\text{Ca}_3(\text{PO}_4)_2$ -epinephrine mixtures was analyzed for epinephrine colorimetrically, using Barker, Eastland and Evers' modification of the Ewin test.<sup>7</sup>

To test the ability of  $\text{Ca}_3(\text{PO}_4)_2$  to adsorb and thus retard the action of procaine, comparisons are planned of the duration of anesthesia produced by plain procaine with that produced by  $\text{Ca}_3(\text{PO}_4)_2$ -procaine mixtures. Preliminary tests are being made on the pressor effect of submucosal injection of epinephrine in peanut oil, which was first introduced by Keeney<sup>8</sup> for the alleviation of asthma.

*Results.* The present experiments, in which 19 dogs were used, confirm the findings of Pickering, *et al.*, with respect to the increase in blood pressure following the submucosal injection of procaine-epinephrine mixtures. The accompanying table shows that the pressor effect of epinephrine injected submucosally is greatly reduced by the addition of colloidal  $\text{Ca}_3(\text{PO}_4)_2$ . In all 3 sites the average percentage increase in blood pressure following the injection of procaine-epinephrine mixtures was greater than that after the injection of epinephrine treated with  $\text{Ca}_3(\text{PO}_4)_2$ . The differences in the two means for each site were found to be statistically significant when analyzed by methods described by Fisher.<sup>9</sup>

$\text{Ca}_3(\text{PO}_4)_2$  with 1:25,000 epinephrine when administered intravenously in dogs gave nearly as great a pressor effect as 2% procaine with 1:25,000 epinephrine. The colorimetric tests on the supernatant liquid from centrifuged colloidal  $\text{Ca}_3(\text{PO}_4)_2$ -epineph-

<sup>7</sup> Barker, J. H., Eastland, C. J., and Evers, N., *Biochem. J.*, 1934, **26**, 2129.

<sup>8</sup> Keeney, E. L., *Am. J. Med. Sci.*, 1939, **198**, 815.

<sup>9</sup> Fisher, *Statistical Methods for Research Workers*.

TABLE I.  
Average Percentage Increase in Blood Pressure Following Submucosal Injections.

Solution	Vol. of injections	No. of animals	Avg % increase in blood pressure		
			Ant. palatine	Mental	Post. palatine
Procaine 2% with epinephrine 1:25,000	2.0 cc	19	43.1	21.9	11.6
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> epinephrine 1:25,000	2.0 "	19	11.5	11.4	4.0

rine mixtures showed that the supernatant fluid contained epinephrine at as great a concentration as did the original solution.

*Conclusions.* It is quite apparent that colloidal Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> diminishes the pressor effect of submucosally injected epinephrine.

### 13185 P

#### Survival of the Respiratory (Gasping) Mechanism in Young Animals Subjected to Anoxia.

W. A. SELLE AND T. A. WITTEN.

*From the Department of Physiology, Medical Branch of the University of Texas, Galveston, Texas.*

Various observers (Reiss and Haurowitz,<sup>1</sup> Avery and Johlin,<sup>2</sup> Kabat and Dennis,<sup>3</sup> Selle and Witten,<sup>4</sup> and Himwich, Alexander and Fazekas<sup>5</sup>) have reported that young animals are much less susceptible to anoxia and asphyxia than adults. The present studies were undertaken to determine whether the primitive respiratory mechanism (gasping) itself survives longer in the young than in the old.

Movements of the mandible of various species (dogs, cats, rabbits, and rats) were recorded mechanically following complete and rapidly induced anoxia produced by ligation of the cerebral vessels or by decapitation. In preliminary experiments it was

<sup>1</sup> Reiss, M., and Haurowitz, F., *Klin. Wchnschr.*, 1929, **8**, 743.

<sup>2</sup> Avery, R. C., and Johlin, J. M., *PROC. SOC. EXP. BIOL. AND MED.*, 1932, **29**, 1184.

<sup>3</sup> Kabat, H., and Dennis, C., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **42**, 534.

<sup>4</sup> Selle, W. A., and Witten, T. A., *Proc. Am. Physiol. Soc.*, 1941, **53**, 253.

<sup>5</sup> Himwich, H. E., Alexander, F. A. D., and Fazekas, J. F., *Proc. Am. Physiol. Soc.*, 1941, **53**, 193.

found that the character and duration of the gasps induced by either of these methods were essentially the same and that the gasping movements of animals in which the cerebral vessels were ligated corresponded to and were simultaneous with labored respiratory movements of the trunk.

It is evident that the respiratory center of the young is much more tenacious and viable than is that of the adult, and that the survival of gasping itself is inversely proportional to age for animals up to or slightly past the weaning period. Thus, the isolated head of a rat 7 weeks of age or older gasps 5 to 8 times over a period of 10 to 20 seconds and remains motionless thereafter; whereas, the head of a younger animal may continue to gasp for many minutes.

Unlike adults, rats under 6 weeks of age display 2 periods of gasping movements: an initial series which consists of 6 to 12 gasps and lasts for a period of 20 to 80 seconds; and a second series which begins after an interval of 30 to 50 seconds following cessation of the initial or first series. The total number of gasps in both series, as well as the total duration of gasping, is greater the younger the animal (as is shown in the table). The second series, which is influenced most by age, lasts 20 to 40 minutes in new-born animals; as age increases, the duration of gasping, as well as the number of gasps, diminishes uniformly in this series. At about the sixth week the second series fails completely.

Gasping is apparently uninfluenced by sensory stimuli from the cut cord and other severed nerve paths, for immersion of the isolated head in 10% formalin, 5% phenol, or 95% alcohol, and cooling of the cord or other cut surfaces with ethyl chloride, do not appreciably alter the nature or duration of gasping; nor does immersion in 5% lactic acid, 5% sodium carbonate or 5% sodium citrate influence gasping.

The survival of the respiratory mechanism seems to parallel other physiological processes as it is further found that (1) pupillary responses of the isolated head, (2) trunk reflexes of the spinal animal, and (3) action of the exposed heart, are all retained longer the younger the animal.

Information is lacking concerning the essential factors responsible for the marked disparity in survival of the respiratory mechanism of young and old animals; metabolism differences, however, appear to be involved.

General anaesthetics (ether, chloroform, and nembutal) alter greatly the strength and duration of the gasps. The first series is completely blocked when surgical anaesthesia is induced prior to decapitation. The second series is also frequently blocked or short-



ened, but in very young animals delayed gasps of reduced amplitude and increased rate occasionally occur.

Morphine tends to obliterate the interval between the first and second series and to reduce slightly the frequency; however, the total duration of gasping, as well as the total number of gasps, is usually greatly increased by this drug.

In very young animals (10 days or less) having a long second series, the frequency of gasping is increased by warming the isolated head and decreased by cooling; the duration of gasping is reduced by warming, increased by cooling. The total number of gasps however, appears to be little altered by temperature unless extreme ranges are employed.

If the area over the medulla and cerebellum of an isolated head of a young animal is quickly cooled by ethyl chloride or by immersion in cold Ringer's, gasping ceases. Upon warming, gasping is resumed and a more or less normal series ensues. Heads of 1 to 4-day-old rats have been revived after more than 30 minutes of cooling.

TABLE I.  
Mandibular Movements of the Isolated Heads of Rats of Various Ages.\*

	Age in days							
	1	2	7	14	21	28	35	90
Gasps in Series I	12.7	13.2	12.3	8.1	7.2	6.9	6.0	5.3
Series II	40.3	30.5	24.0	19.5	9.2	4.9	2.3	0.0
Total gasps	53.0	43.7	36.3	36.3	16.4	11.8	8.3	5.3
Total duration, sec	1630	1450	745	278	117	75	56.3	14.5

\*Values represent averages for 10 animals.

13186

### Immunizing Capacity of Virus of Eastern Equine Encephalomyelitis Inactivated by Ultraviolet Light.

ISABEL M. MORGAN AND GEORGE I. LAVIN.  
(Introduced by Peter K. Olitsky.)

*From the Laboratories of the Rockefeller Institute for Medical Research, New York City.*

The inactivating effect of ultraviolet light on viruses as well as bacteria has been observed repeatedly. That viruses so inactivated may be effective as immunizing antigens has already been shown for at least 2 viruses capable of causing disease in man. With a



Cerebral Resistance to E.E.E. Virus in Mice  
after Vaccination with Virus Inactivated  
by Means of Ultraviolet Light (U.V.) or Formalin (F.V.)

Virus dilution	Mice: Vaccinated		Virus dilution	Non-vaccinated
	U.V.	F.V.		
$10^{-2}$	■ ■ ■ ■	■ ■ ■ □	$10^{-7}$	■ ■ ■ ■ ■
$10^{-3}$	□ □ □ □	■ ■ □ □ □	$10^{-8}$	■ ■ ■ ■ □
$10^{-4}$	■ ■ □ □ □	■ ■ □ □ □	$10^{-9}$	■ □ □ □ □

■ = 1 mouse died      □ = 1 mouse survived

FIG. 1.

vaccine prepared by irradiating mouse-brain infected with rabic virus, Webster and associates<sup>1</sup> successfully immunized mice and dogs against a subsequent injection of active virus. Salk, Lavin, and Francis<sup>2</sup> compared the antigenic potency of epidemic-influenza virus following irradiation with that of active virus. In high concentrations, irradiated virus was nearly as effective an immunizing antigen as active virus; when lower concentrations were tested, a hundredfold loss in immunizing capacity was found to have occurred during irradiation. Ultraviolet light has been applied to the virus of equine encephalomyelitis, Eastern strain (E.E.E.), by Sharp and associates;<sup>3</sup> they studied the molecular stability of ultraviolet-treated virus. The preparation of an immunizing antigen produced by irradiation of E.E.E. virus with ultraviolet light is reported here.

Chick embryos 7-days-old were inoculated with 0.1 cc  $10^{-8}$  suspension of E.E.E. virus-infected embryo in 0.85% saline solution. Embryos removed from the egg 18-20 hours after inoculation were rinsed in saline solution, ground in a mortar and made to a 10% suspension in saline or Tyrode's solution. This suspension was centrifuged at 2000 rpm for 10 minutes; the centrifugation was repeated with the supernate; the supernate then obtained was spun in a Swedish angle-centrifuge at 4000 rpm for 45 minutes. About

<sup>1</sup> Hodes, H. L., Lavin, G. I., and Webster, L. T., *Science*, 1937, **86**, 447; Webster, L. T., and Casals, J., *J. Exp. Med.*, 1941, **73**, 601.

<sup>2</sup> Salk, J. E., Lavin, G. I., and Francis, T., *J. Exp. Med.*, 1940, **72**, 729.

<sup>3</sup> Sharp, D. G., Taylor, A. R., Beard, D., Finkelstein, H., and Beard, J. W., *Science*, 1940, **92**, 359.

30 cc of the final supernate were transferred to a quartz test-tube, with an internal diameter of 2.1 cm, which was placed in the center of a quartz-mercury resonance lamp in the form of a spiral\* with an internal diameter of 9 cm. The spiral, 15 cm in height, consists of 7 coils. The tube containing viral suspension was so placed that there was at least one complete coil of the lamp above and below the suspension. About 85% of the energy emitted by this type of lamp is in the line of 2537 Ångström units. The lamp operates at 30 milliamperes and 15,000 volts transformed from 110 volts A.C. The titer of virus before irradiation was regularly  $10^8$  when injected intracerebrally in albino mice, Rockefeller Institute strain. After beginning of irradiation, samples of virus were removed at 5-minute intervals; the suspension was stirred at each sampling; 0.03 cc of each sample was tested for infectivity by intracerebral injection of each of 4 mice. Inactivation was complete after 15-25 minutes of irradiation.

The immunizing capacity of E.E.E. virus inactivated by ultraviolet light was compared with that of formalin-inactivated virus prepared from the same viral suspension, since formalin-inactivated virus has come to be accepted as a standard immunizing antigen. In Fig. 1 the result of one experiment is charted. A group of 11 mice was vaccinated by means of 3 intraabdominal injections on alternate days of 0.25 cc 10% suspension inactivated by 20 minutes' irradiation. Another group was vaccinated with similar doses of virus inactivated with 0.5% formalin. Non-vaccinated mice served as controls. Two weeks after beginning of vaccination, all mice were injected intracerebrally with a broth-suspension of mouse-brain infected with E.E.E. virus in dilutions indicated in Fig. 1. At least half of each group of vaccinated mice survived 10,000 and 100,000 cerebral units. There was no difference between the two vaccinated groups in the degree of immunity induced. It should be added that the antigenicity of such irradiated E.E.E. virus was found to diminish rapidly with further irradiation, an effect already noted for rabic virus.<sup>1</sup>

In summary, a non-infective immunizing antigen has been produced by ultraviolet irradiation of the virus of Eastern equine encephalomyelitis.<sup>4</sup>

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\* Manufactured by the Hanovia Chemical Company.

<sup>4</sup> Drs. Casals and Palacios find that E.E.E. virus inactivated by ultraviolet light serves as a highly specific complement-fixing antigen against anti-E.E.E. serum. Their results will be published.

### New Observation on a Primary Ocular Reaction to Shwartzman Toxins.

CORRADO AJÓ. (Introduced by G. Shwartzman.)

*From the Laboratories, Mount Sinai Hospital, New York City.*

This report deals with a marked reaction in the eye of the rabbit following the intravenous injection of Shwartzman toxins.\* It consists of miosis, photophobia, lacrimation, congestion of the iris and conjunctiva with a marked pericorneal ring of dilated capillaries and in some instances with gross conjunctival hemorrhages. Ophthalmoscopic examination is rendered difficult by the turbidity of the dioptic media although a marked congestion of the fundus may be revealed. The ciliary bodies show enhanced permeability to fluorescein injected intravenously. The aqueous humor of the anterior chamber is usually under high pressure and coagulates immediately upon removal. The fluid is clear in appearance although numerous fibrin-threads, crystals, and epithelial cells may be seen microscopically. The reaction reaches its maximal intensity within 2 hours following the intravenous injection of potent toxins but it completely disappears 24 hours later. The toxins may be given intravenously and intraabdominally, the cutaneous route is only successful when a vascularized area of the skin is used (the upper third of the ear). Microscopically, severe reactions show massive conjunctival hemorrhage with dilatation and engorgement of blood vessels of the recti muscles. No lesions have been seen in the iris, retina, cornea, and ciliary bodies.

Incidentally, other primary toxic effects following the intravenous injection of the Shwartzman filtrates may be noted, *i. e.*, severe diarrhea, enhanced fragility of the capillaries of the skin, serous fibrinous exudation in the joints, some enhanced permeability of the choroid plexus to fluorescein and a high mortality-rate. These effects may be considerably diminished by a previous local preparation of rabbits to the Shwartzman phenomenon (observed on about 50 animals). No similar protection is afforded by local preparation with chemical irritants and inflammatory agents devoid of Shwartzman principles (cantharidin plaster, turpentine, diphtheric toxin, etc.).

Two intravenous injections of 100 reacting units of Shwartzman

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\* *i.e.*, bacterial factors capable of producing the Shwartzman phenomenon.

toxins 24 hours apart produce very severe ocular reactions as well as enhancement of other toxic effects.

The observations were made on a total of 300 albino male rabbits (average weight 2.5 kg). The ocular reaction was obtained in 95 out of 100 rabbits injected intravenously with "agar-washings" culture-filtrates of meningococcus Type III and *B. typhosus* and their dialysates through cellophane No. 600. It failed to appear in 100 control rabbits injected intravenously with similar or larger doses of bacterial filtrates devoid of Shwartzman reacting potency and various non-bacterial substances, *i. e.*, diphtheric toxin, staphylococcus Type A, *Streptococcus viridans*, sterile "agar-washings" filtrates, human, pig, horse, and ox sera in 1% solution, 2% suspension of silicic acid, etc.

Pooled aqueous humor obtained from 5 rabbits with severe ocular reactions when injected intravenously into normal rabbits produced fever, diarrhea, ocular reactions and in previously locally prepared rabbits—the Shwartzman phenomenon. Neutralization experiments described below were carried out in order to demonstrate the fact that the ocular reactions were due to the toxic principles of the filtrates.

Normal rabbits received typhoid toxin variously diluted in saline. The highest dilution (1:640) of toxin giving only doubtful coagulation of the aqueous humor 2 hours after the intravenous injection was chosen as a toxin-unit.

One cc of an antityphoid horse sera (capable of neutralizing the Shwartzman phenomenon) was mixed with various amounts of typhoid toxin, incubated at 37°C for one hour, and injected intravenously into normal rabbits. One to 10 units of toxin titrated as above failed to produce the ocular reaction or coagulation of the aqueous humor.

To sum up, the experiments demonstrate a severe reaction in the eye of rabbits following the intravenous injection of Shwartzman toxins into the general circulation. It is accompanied by enhanced capillary permeability and other damage. The factors in the toxic filtrates responsible for the eye reaction may be inactivated by immune neutralizing serum.



### Virus Distribution in Reversible Soluble and Insoluble Phases of Neutralized Papilloma-Virus Protein.\*

DOROTHY BEARD, D. G. SHARP, A. R. TAYLOR, AND J. W. BEARD.

*From the Department of Surgery, Duke University School of Medicine,  
Durham, N.C.*

The neutralizing effect of homologous immune serum on the rabbit papilloma-virus protein is quantitatively reversible<sup>1</sup> by simple dilution over the whole range<sup>2</sup> of serum-virus relations accessible to study by infectivity-measurement.<sup>3</sup> Under proper conditions of serum-virus proportions, precipitates occur in the mixtures, consisting presumably of virus and specific antibody. In the present study the distribution of virus between the soluble and insoluble phases in one region of serum-virus quantities has been determined.

The results reported here were derived from 8 correlated experiments with papilloma-virus protein and an antipapilloma rabbit-serum designated as D. R. 496. The findings of 7 experiments with this serum have been tabulated in detail elsewhere.<sup>1, 2</sup> An additional study made in the desired region of serum-virus relations is described here.

Papilloma-virus protein procured under standard conditions<sup>3</sup> by ultracentrifugation was mixed with undiluted immune serum D. R. 496 as previously reported<sup>2</sup> so that the final concentration of total virus was 24.4  $\gamma$  per 0.1 cc (virus pI 4.6, serum pD 0).<sup>1, 2</sup> After 1½ hours the precipitate forming at room-temperature was sedimented in an ordinary horizontal International Centrifuge No. 2 at full speed for 30 minutes. The clear supernatant fluid was carefully pipetted from the pellet which was then suspended in 0.9% NaCl solution to the volume of the supernatant fluid (4.5 cc). The supernatant fluid and the pellet-suspension were inoculated<sup>3</sup> in successive twofold dilutions in a group of 32 rabbits, a number statistically adequate<sup>3</sup> for the problem at hand. Twofold dilutions of untreated viral protein were inoculated in the same animals for standardization.

With the serum-virus relations described, the free virus of the

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\* This work was aided by the Dorothy Beard Research Fund and by a grant from Calco Chemical Co., Bound Brook, N.J.

<sup>1</sup> Bryan, W. R., and Beard, J. W., *J. Infect. Dis.*, 1941, **68**, 133.

<sup>2</sup> Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Infect. Dis.*, in press.

<sup>3</sup> Bryan, W. R., and Beard, J. W., *J. Infect. Dis.*, 1939, **65**, 306.



whole mixture without separation of the precipitate is 3  $\gamma$  per 0.1 cc as previously reported,<sup>2</sup> indicating neutralization of 21.4  $\gamma$  of the 24.4  $\gamma$  initially present. When the precipitate was sedimented, however, the free virus in the supernatant fluid is not 3  $\gamma$  but 0.008  $\gamma$ . Dilution of the pellet-suspension resulted in progressive freeing of virus until the seventh twofold dilution where the rate of change in free virus paralleled that of the untreated viral preparations containing equivalent amounts of total virus. From the relation of control and experimental regression lines<sup>1, 3</sup> in this region, the total virus present in the initial pellet suspension of 4.5 cc was found to be 6.1  $\gamma$  per 0.1 cc. Of this, 0.9  $\gamma$  per 0.1 cc was in free form. In the supernatant fluid, then, there was 18.3  $\gamma$  total virus of which 99.1% was present as soluble neutralized virus. This represented approximately 75% of the total virus per 0.1 cc initially used.

A possible explanation of the above facts may lie in the following equilibria between virus and serum. Virus and serum mixed under the present conditions yield a system of soluble and insoluble phases of neutralized virus. With the system intact, *i. e.*, prior to centrifugation, equilibrium can be expressed as



It is known that the state of equilibrium for a given concentration of serum and virus is constant<sup>1, 2</sup> and that the equilibrium is reversible as shown by the dilution-phenomenon in the present and previous experiments.<sup>1, 2</sup> Under these circumstances the combined virus in both soluble and insoluble phases will contribute to the level of free virus in the intact system. When the precipitate is sedimented, however, the effect of the insoluble phase will be reduced by removing it from contact with the soluble phase, resulting in further combination of free virus and free serum to satisfy the equilibrium. Such is possible, for free virus exists in the uncentrifuged mixture in the amount of 3  $\gamma$  per 0.1 cc, as determined by direct measurements<sup>1, 2</sup> and the intact system contained enough serum to neutralize a total of 27.7  $\gamma$  virus per 0.1 cc in the presence of an optimal quantity<sup>1</sup> of virus, 44.9  $\gamma$  per 0.1 cc. In the end, the level of free virus in the supernatant fluid will depend on the demands for equilibrium between free virus and the soluble virus-serum complex, and thus the quantity of free virus in the supernatant fluid is reduced below the level existing in the intact system. The amount of free virus found in the pellet-suspension diluted to 4.5 cc was related at least in part to dissociation by dilution, so that it cannot

<sup>†</sup>V—virus; B—antibody; (V.B.)—soluble phase; and (V.B.)<sub>↓</sub>—insoluble phase.

be said whether or not all of the virus precipitated was in the neutralized state.

These results provide direct evidence for the existence of both soluble and insoluble phases of neutralized virus and for the dissociation of both with dilution when the 2 phases are separated. The data likewise afford an estimate of the distribution of virus between the 2 phases separated in this way by centrifugation. It should be emphasized that the described distribution can be considered only for the special region of serum-virus amounts employed. The reduction in free virus in the supernatant fluid resulting from removal of the insoluble phase, a phenomenon already noted by others in studies with unpurified viral preparations,<sup>4</sup> could not have been due to sedimentation of free virus since it was not demonstrable in the pellet-suspension. The findings suggest the possibility of a shift in chemical equilibrium as an explanation of the phenomenon.

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#### Comparative Activity of Nicotinic Acid and Nicotinamide as Growth Factors for Microorganisms.\*

STEWART A. KOSER, SAM BERKMAN AND ALBERT DORFMAN.

*From the Departments of Bacteriology and Parasitology and of Biochemistry, University of Chicago.*

Investigations of the past few years have demonstrated that a number of microorganisms must be supplied with either nicotinic acid or nicotinamide for continued cell multiplication. When these compounds are supplied in excess no difference in activity is noted, but when suboptimum quantities are used a quantitative difference in activity is often encountered.

While studying the nutritive requirements of the *Pasteurella* group of bacteria, it was found that nicotinamide could *not* be replaced by nicotinic acid in the cultivation of those members of the *Pasteurella* group which cause hemorrhagic septicaemia in animals. In a basal medium of hydrolyzed purified gelatin, supplementary amino acids, glucose and inorganic salts, growth of many strains of the hemorrhagic septicaemia *Pasteurellae* occurs readily when both

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<sup>4</sup> Friedewald, W. F., and Kidd, J. G., *J. Exp. Med.*, 1940, **72**, 531.

\* This investigation was aided by grants from the John and Mary R. Markle Foundation and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

nicotinamide and pantothenic acid are added.<sup>1</sup> On substitution of nicotinic acid for nicotinamide growth of cultures does not occur. This observation has been repeated a number of times with 16 *Pasteurella* cultures. Two different samples of both nicotinic acid and nicotinamide were used. One-tenth microgram of nicotinamide per cc of medium is sufficient for prompt growth of cultures whereas amounts of 0.1, 1.0, 10.0 and 100  $\mu\text{g}$  of nicotinic acid per cc, in the presence of pantothenic acid, did not cause growth. The same samples of nicotinic acid supported growth of dysentery bacilli and *Proteus*.

The inability of the hemorrhagic septicaemia *Pasteurellae* to utilize nicotinic acid was shown further by metabolic studies of a typical *Pasteurella* culture. The culture used for these experiments was grown in the basal medium of hydrolyzed gelatin previously described<sup>1</sup> supplemented with 0.004  $\mu\text{g}$  per cc of nicotinamide and 0.1  $\mu\text{g}$  per cc of calcium pantothenate. The amount of nicotinamide used was suboptimum but permits the development of a crop of cells sufficient for the tests. Cells from 24-hour cultures at 37°C were centrifuged, washed with *M*/20 phosphate buffer at pH 7.4, recentrifuged and suspended in the phosphate buffer.

Comparative data are given in Tables I and II. The figures shown are typical of other tests. Both diphosphopyridine nucleotide and nicotinamide stimulated respiration of *Pasteurella suisseptica* grown in a medium deficient in nicotinamide, while nicotinic acid produced no such stimulation. Other tests have shown that pantothenic acid together with either nicotinamide or nicotinic acid gave results equivalent to those secured with the amide or the acid alone. These results confirm the growth tests in showing that certain members of the *Pasteurella* group are able to utilize nicotinamide but not nicotinic acid.

TABLE I.  
Effect of Nicotinamide, Nicotinic Acid and Diphosphopyridine Nucleotide on Methylene Blue Reduction by *Pasteurella suisseptica*.

	Time required for reduction,* min
Control	>60
Nicotinamide (20 $\gamma$ )	20
Nicotinic acid (20 $\gamma$ )	>60
Diphosphopyridine nucleotide (50 $\gamma$ )	17

\*Each Thunberg tube received 0.3 cc of 2% glucose and 2 cc of a suspension of washed cells in phosphate buffer. After 20 minutes' equilibration at 38°C 50  $\gamma$  of methylene blue were tipped into each tube.

<sup>1</sup> Berkman, S., Saunders, F., and Koser, S. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **44**, 68.

TABLE II.

Effect of Nicotinamide, Nicotinic Acid and Diphosphopyridine Nucleotide on Respiration of *Pasteurella suisepitica* as Measured by the Direct Warburg Method.

	Oxygen consumed in 2 hr,* mm <sup>3</sup>
Control	13.0
Nicotinamide (20 γ)	69.0
Nicotinic acid (20 γ)	16.3
Diphosphopyridine nucleotide (50 γ)	161.7

\*Each vessel received 0.3 cc of a 2% solution of glucose and 2 cc of a suspension of washed cells in phosphate buffer. After 10 minutes' equilibration at 38°C the substance being tested was tipped into the vessel from the sidearm. The total volume in each vessel was 2.7 cc.

In Table III the relative growth-promoting effects of nicotinamide and nicotinic acid for several different microorganisms have been tabulated. The reports are arranged in the order of increasing effectiveness of the amide. The results of our present study are included in the last line of the table.

This arrangement brings out strikingly the relative effectiveness of the amide and acid in promoting growth of those microorganisms for which these compounds must be supplied. The finding for the diphtheria bacillus, showing a distinctly greater growth-promoting effect for the acid, is quite different from that of other microorganisms thus far reported. *Proteus* can apparently make use of the acid about as effectively as the amide, while the staphylococcus

TABLE III.

Relative Growth-promoting Effect of Nicotinamide and Nicotinic Acid.

Organism	Ratio of activity, amide to acid*
Diphtheria bacillus <sup>2</sup>	1:10
<i>Proteus</i> <sup>3</sup>	1:1
<i>Proteus</i> <sup>4</sup>	1:1
<i>Staphylococcus</i> <sup>5</sup>	5:1
Dysentery bacillus <sup>6</sup>	10:1
Certain <i>Pasteurellæ</i>	∞ (acid ineffective)

\*The results given in the literature are usually on the basis of growth at the first 24 or 48 hours after inoculation. In at least one instance, however (dysentery bacillus), it has been shown that on continued incubation of cultures for 7 to 14 days, slow growth occurs in the presence of successively smaller amounts of nicotinic acid, so that eventually the results with the two compounds are much the same. In this instance, the results at 24 hours are significant from the standpoint of bringing out differences in growth-promoting effect.

<sup>2</sup> Mueller, J. H., *J. Bact.*, 1937, **34**, 429.

<sup>3</sup> Fildes, P., *Brit. J. Exp. Path.*, 1938, **19**, 239.

<sup>4</sup> Breslove, B. B., unpublished results from this laboratory.

<sup>5</sup> Knight, B. C. J. G., *Biochem. J.*, 1937, **31**, 731.

<sup>6</sup> Dorfman, A., Koser, S. A., Reames, H. R., Swingle, K. F., and Saunders, F., *J. Infect. Dis.*, 1939, **65**, 163.



and dysentery bacilli evidently experience more difficulty in the utilization of the acid. While due allowance must be made for variation of individual strains the general trend is quite evident.

When nicotinic acid is supplied in place of the amide, it is often assumed that in the course of cell metabolism the acid is first converted to the amide before the compound is utilized further in cell respiration, although the results with the diphtheria bacillus would seem to be an exception to this interpretation. From the data presented it appears that microorganisms differ markedly in their ability to bring about this conversion. In view of these differences it seems reasonable to expect the existence of microorganisms which are completely unable to bring about this conversion and thus require the amide without being able to utilize the acid. The hemorrhagic septicaemia *Pasteurellae* appear to fit in this category and represent the widest divergence in ability to utilize these two compounds.

A further summary relating to the entire enzyme-coenzyme complex is of interest in showing gradations in synthetic properties among microorganisms. *Bacterium coli* may be taken as an example of those organisms which can synthesize the entire diphosphopyridine nucleotide molecule. Most strains of dysentery bacilli, *Proteus* and *Staphylococcus* are unable to synthesize the pyridine moiety of the nucleotide. These organisms, when supplied nicotinic acid, differ quantitatively in their ability to convert the acid to the amide but nevertheless are all able to perform this reaction and are also able to synthesize the coenzyme. Certain *Pasteurellae* are unable to convert the acid to the amide, but are able to synthesize the coenzyme when the amide is supplied, while *Hemophilus influenzae* must be supplied with the whole coenzyme.<sup>†</sup> It is possible that some of the intracellular parasites represent a further step in this series, and must be supplied with the enzyme as well as the coenzyme.

*Summary.* Nicotinic acid and nicotinamide do not always produce equivalent growth-promoting effects upon microorganisms. A series of microorganisms is presented showing a ratio of activity of amide to acid varying from one-tenth to infinity. The last case is represented by certain organisms of the *Pasteurella* group whose growth and respiration is promoted by nicotinamide, but not by nicotinic acid. A further summary is presented of synthetic abilities of different microorganisms with respect to the entire enzyme-coenzyme complex.

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<sup>†</sup> While it is commonly assumed that the whole coenzyme molecule is needed by *H. influenzae*, it should be pointed out that the pyridine-ribose-phosphate portion of this molecule has never been tested for V factor activity. See Bass *et al.*, *J. Infect. Dis.*, 1941, **68**, 175.



## Chemotherapeutic Evaluation of Some N<sup>1</sup>- and N<sup>4</sup>-Heterocyclic Derivatives of Sulfanilamide.

FRANK B. COOPER, PAUL GROSS, AND MARION LEWIS.

*From the Western Pennsylvania Hospital Institute of Pathology, Pittsburgh, Pa.*

The clinical success which has attended the use of sulfapyridine, and more recently, sulfathiazole, has stimulated an active interest in the heterocyclicsulfanilamide derivatives as chemotherapeutic agents. A review of the compounds of this class which have been tested against experimental infections lists the following groups of nuclear-coupled N<sup>1</sup>-heterocyclicsulfanilamides other than those of pyridine and thiazole: those of tetrazole,<sup>1</sup> 1,3,4-thiadiazole,<sup>1</sup> pyrroline,<sup>2</sup> pyrrolidine,<sup>2</sup> 5-pyrazolone,<sup>1</sup> pyrimidine,<sup>1</sup> benzothiazole<sup>1</sup> and quinoline.<sup>3</sup> Among the N<sup>1</sup>-acylheterocyclics were the furoyl- and nicotinyl-derivatives.<sup>4</sup>

The substitution of halogen in the 5-position of 2-sulfanilamidopyridine (sulfapyridine) destroyed its activity, whereas substitution of nitro or amino groups in this position was claimed to enhance the antistreptococcic activity, but slightly depress the antipneumococcic activity.<sup>5</sup> The substitution of an amino group in the 6-position left the activities practically unaltered.<sup>3,6</sup> 3-Sulfanilamidopyridine<sup>5</sup> and its 6-chlor<sup>5</sup> and 6-brom<sup>5</sup> derivatives were as active as sulfapyridine, the 6-ethoxy derivative was slightly active,<sup>5</sup> the 6-amino<sup>5</sup> and 6-hydroxy<sup>5</sup> derivatives, inactive. The substitution of halogen in the 2-position of 5-sulfanilamidopyridine resulted in an active compound,<sup>5</sup> whereas the corresponding amino and nitro compounds were inactive.<sup>5</sup>

The therapeutic activity of 2-sulfanilamidothiazole (sulfathiazole) has, with one exception, been more sensitive to the effects of substitution than that of sulfapyridine. Substitution of a methyl group in the 4-position (sulfamethylthiazole) did not affect its antistreptococcic,<sup>6,7</sup> antipneumococcic,<sup>6,7</sup> or antistaphylococcic ac-

<sup>1</sup> Roblin, R. O., Jr., Williams, J. H., Winnek, P. S., and English, J. P., *J. Am. Chem. Soc.*, 1940, **62**, 2002.

<sup>2</sup> Cass, W. E., *J. Am. Chem. Soc.*, 1940, **62**, 3255.

<sup>3</sup> Tudur, K., Itikawa, A., and So, D., *J. Pharm. Soc. Japan*, 1939, **59**, 213.

<sup>4</sup> Crossley, M. L., Northey, E. H., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1939, **61**, 2950.

<sup>5</sup> Roblin, R. O., Jr., and Winnek, P. S., *J. Am. Chem. Soc.*, 1940, **62**, 1999.

<sup>6</sup> Cooper, F. B., Gross, P., and Lewis, M., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 421.

<sup>7</sup> Barlow, O. W., and Homburger, E., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 317.

tivity<sup>8, 9</sup> appreciably, although the substitution of a phenyl group in the 4-position diminished these activities.<sup>7, 8</sup>

The other groups of N<sup>1</sup>-heterocyclic derivatives possess little or no therapeutic action, except 2-sulfanilamidopyrimidine (sulfadiazine). This compound is claimed to be as active as sulfapyridine and sulfathiazole against streptococcic,<sup>1, 10</sup> pneumococcic<sup>1, 10</sup> and staphylococcic<sup>1, 10</sup> infections, and considerably more active than either drug against experimental infections with Friedländer's bacillus.<sup>10</sup> The 4-methyl derivative, 2-(sulfanilamido)-4-methylpyrimidine, is reported to be as active as the parent sulfadiazine, whereas 4-sulfanilamidopyrimidine and 5-sulfanilamidouracil are inactive.<sup>1</sup>

N<sup>4</sup>-2-pyridylsulfanilamide and N<sup>4</sup>-2-quinylsulfanilamide were inactive against streptococcic and pneumococcic infections,<sup>11</sup> whereas N<sup>4</sup>-bromotetronylsulfanilamide was claimed to be as active as sulfanilamide against streptococcic infections.<sup>12</sup>

Of the N<sup>4</sup>-heterocyclic-acyl-sulfanilamides, the 2-furoyl<sup>13</sup> and 2-thenoyl derivatives<sup>13</sup> showed decreased activity; N<sup>4</sup>-quinolinylsulfanilamide, activity equal to that of sulfanilamide<sup>14</sup> and N<sup>4</sup>-(5-pyrrolidone-2-carbonyl)-sulfanilamide, even greater activity.<sup>15</sup> N<sup>4</sup>-nicotinylsulfanilamide was claimed to be both more active<sup>16</sup> and less active than sulfanilamide.<sup>13</sup>

In the present study a number of previously unreported heterocyclic derivatives are compared to sulfapyridine, sulfathiazole, and sulfadiazine against experimental infections of mice. All mice dying within the 21-day period were autopsied and those not dying of the given infection were discarded from the series.

In the antistreptococcic and antipneumococcic evaluations, treatment was withheld for 3 hours to allow infection to become established. This method of assay was considered preferable to the prophylactic method of Bieter and co-workers,<sup>17</sup> in which the drug is

<sup>8</sup> Barlow, O. W., and Homburger, E., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **42**, 792.

<sup>9</sup> Rake, G., and McKee, C. M., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 561.

<sup>10</sup> Feinstone, W. H., Williams, R. D., Wolff, R. T., Huntington, E., and Crossley, M. L., *Bull. Johns Hopkins Hosp.*, 1940, **67**, 427.

<sup>11</sup> Gray, W. H., *J. Chem. Soc.*, 1939, 1202.

<sup>12</sup> Kumler, W. D., *J. Am. Chem. Soc.*, 1940, **62**, 2560.

<sup>13</sup> Kolloff, H. G., and Hunter, J. H., *J. Am. Chem. Soc.*, 1940, **62**, 1646.

<sup>14</sup> Hykes, O. V., Hykes, D. E., and Rerabek, J., *Compt. rend. Soc. de biol.*, 1937, **126**, 635.

<sup>15</sup> Gray, W. H., Buttle, G. A. H., and Stephenson, D., *Biochem. J.*, 1937, **31**, 724.

<sup>16</sup> Daniels, T. G., and Iwamoto, H., *J. Am. Chem. Soc.*, 1940, **62**, 741.

<sup>17</sup> Bieter, R. D., Larson, W. P., Cranston, E. M., and Levine, M., *J. Pharm. and Exp. Therap.*, 1939, **66**, 3.

TABLE I.  
Streptococcal Infection of Mice

Treatment	No. of Mice	No. of deaths daily during 21 days										No. of Survivors	% Survivors	Efficacy of each drug compared to that of every other drug*									
		1	2	3	4	5	6	7	8	9	21			I	H	G	F	E	D	C	B	A	
None	45	43		1		1						None	None	0	—	0	—	—	—	—	—	—	—
A Sulfapyridine	44											33	75	+	0	+	+	+	+	+	+	0	—
B Sulfathiazole (1)	43	1										26	61	+	0	+	+	0	+	0	+	0	—
C 2-(Sulfanilamido)-5-ethyl-4-thiazolone (2)	43																						
D 2-(Sulfanilamido)-4, 5, 6, 7-tetrahydrobenzothiazole (1)			1		1	7	3	6	2	2		21	49	+	0	+	+	0	+	0	+	0	—
E 2-(Sulfanilamido)-5-methyl-1, 3, 4-thiadiazine (1)	20	1										3	15	0	+	0	+	—					
F N <sup>4</sup> -Nicotinylsulfathiazole (1)	30																						
G N <sup>4</sup> -Acetylsulfamyl-2-methyl-4, 5-dihydroimidazole (3)	15	11	3									17	57	+	0	+	+						
H 2-(4-Nitrobenzenesulfonamido)thiazole (1)	20	19	1									None	None	0	—								
I 2-(Sulfanilamido)-6-amino-isonicotinic acid (4)	20											11	35	+									
	20	20										None	None										

*Infection:* 0.5 cc of a 10-4 broth dilution of an 18-hr broth culture (Richards) intraperitoneally (1000 fatal doses).  
*Treatment:* 10 mg of drug suspended in 0.2 cc of 15% gum acacia orally 3 hours after infection, then once daily for 3 successive days (total 40 mg). At time of first treatment, blood cultures of 6 mice, selected at random from the series, averaged more than 7 colonies per cc.

Drugs were synthesized and donated by the (1) Maltbie Chemical Company, Newark, N. J.; (2) Sharp and Dohme, Glenolden, Pa.; (3) Monsanto Chemical Company, St. Louis, Mo.; and (4) Charles Pfizer and Company, Inc., New York, N. Y.

\*The drugs in the left hand column are evaluated against those designated by letters A to I along the top by the symbols: +: better than; —: worse than; and 0: not significantly different from. This table was derived by the extraction of  $\chi^2$  from the original data. P values less than 0.05 were accepted as evidence of valid difference. Where significant difference occurs, "better than" or "worse than" were determined by inspection of original data.

administered in the ration for 48 hours before infection, because it more closely approximates clinical conditions, namely, the treatment of an established infection.

*Streptococcal Infection.* Table I shows that the 3 compounds: 2-(sulfanilamido)-5-methyl-1,3,4-thiadiazine, 2-(4-nitrobenzenesulfonamido)-thiazole and 2-(sulfanilamido)-5-ethyl-4-thiazolone (sulfaethylthiazolone) have activities comparable to that of sulfa-thiazole, but not as pronounced as that of sulfapyridine.

*Pneumococcal Infection.* Since the activity of sulfathiazole has been shown to be practically equal to that of sulfapyridine,<sup>6, 10, 18, 19</sup> the latter drug was used as a standard of comparison. Table II shows that sulfaethylthiazolone is practically as active as sulfapyridine.

*Staphylococcal Infection.* Since sulfaethylthiazolone showed adequate antistreptococcal and antipneumococcal activity, its therapeutic action was compared to that of sulfathiazole and sulfadiazine against staphylococcal infections. Reference to Table III shows that all 3 drugs possess comparable activity.

In considering the correlation between structure and therapeutic activity, it is generally accepted that the active phenylsulfonamides either possess a free N<sup>4</sup>-amino group or a structure which is capable of yielding this group *in vivo*. However, the converse is not necessarily true. The activity of 2-(4-nitrobenzenesulfonamido) thiazole is accounted for by the fact that blood levels of 1.9, 3.6, and 2.8 mg % of diazotizable material, calculated as sulfathiazole, were observed in mice one, 4, and 7 hours, respectively, after the oral administration of 20 mg. Traces were observed at the end of 24 hours. The inactivity of nicotinylsulfathiazole is explained by the fact that mice which received 20 mg orally failed to show during 24 hours any appreciable free or conjugated diazotizable material which could be detected by the Bratton and Marshall<sup>20</sup> method, or our modification, which gives significantly higher recovery of free and conjugated sulfathiazole.<sup>21</sup>

Toxicity studies showed sulfaethylthiazolone to be less acutely toxic than sulfapyridine or sulfathiazole. Of 8 mice which received 25 mg orally twice daily until the time of death, 6 died between the eleventh and twenty-third days and 2 survived. Blood counts made

<sup>18</sup> McKee, C. M., Rake, G., Greep, R. O., and van Dyke, H. B., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 417.

<sup>19</sup> Litchfield, J. T., White, J. H., and Marshall, E. K., Jr., *J. Pharm. and Exp. Therap.*, 1940, **69**, 166.

<sup>20</sup> Bratton, C. A., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

<sup>21</sup> Cooper, F. B., Gross, P., and Lewis, M., in press.

TABLE II. Pneumococci Infection of Mice.

Treatment	No. of Mice	No. of deaths daily during 21 days											No. of Sur- vivors	% Sur- vivors	Efficacy of each drug compared to that of every other drug*							
															I	H	F	E	C	A		
		1	2	3	4	5	6	7	8	9-21												
None	75	14	49	12									None	None	0	—	0	—	—	—		
A Sulfapyridine	73	2	6	1	4	2	19	4	8				27	37	+	0	+	0	0	0		
C 2-(Sulfanilamido)-5-ethyl-4-thiazolone (2)	73	1	5	6	12	2	7	4	17				19	26	+	0	+	0				
E 2-(Sulfanilamido)-5-methyl-1,3,4-thiadiazine (1)	15	2	4	3	4								2	13	0	0	0	0				
F N <sup>4</sup> -Nicotinylsulfathiazole (1)	15	7	8										None	None	0	0	0					
H 2-(4-Nitrobenzenesulfonamido)thiazole (1)	15	1	1	2	5	1	3						2	13	0							
I 2-(Sulfanilamido)-6-amino-isonicotinic acid (4)	15	1	9	2	2	1							None	None								

*Infection:* 0.5 cc of a 10-5 broth dilution of an 18-hr broth culture (Binda, Type II) subcutaneously (650 fatal doses).

*Treatment:* 20 mg of drug suspended in 0.2 cc of 15% gum acacia orally 3 hours after infection, then once daily for 5 successive days (total 120 mg).

(1) (2) (4)\* See Legend of Table I.

TABLE III. Staphylococci Infection of Mice.

Treatment	No. of Mice	No. of deaths daily during 21 days											No. of Survivors	% Survivors	Efficacy of each drug compared to that of every other drug*			
															J	C	B	
		1	2	3	4	5	6	7	8	9-21								
None	71	6	12	12	3	4	2	2	3				15	21	—	—	—	
B Sulfathiazole (1)	64	1			1	1	1	1	12				48	75	0	0		
C 2-(Sulfanilamido)-5-ethyl-4-thiazolone (2)	64	1	2	1	2	2	1		3	14			38	59		0		
J Sulfadiazine	65	1	2	3	5	2	8						44	68				

*Infection:* 0.2 cc of a broth suspension from an 18-hr blood agar culture (F.D.A. 209) intravenously (160 million cocci).

*Treatment:* 10 mg of drug suspended in 0.2 cc of 15% gum acacia orally at time of infection and 6 hours later, then twice daily for 4 days, followed by single daily treatment for 5 days (total 150 mg). (1) (2)\* See legend of Table I.

We wish to thank Dr. D. A. Bryce, of Lederle Laboratories, Inc., New York, N. Y., for supplying the sulfadiazine; and Dr. O. W. Barlow, of Winthrop Chemical Co., Inc., New York, N. Y., for supplying culture F.D.A. 209.



every second day showed a mean fall in erythrocytes from 9,200,000 to 5,821,000 during 16 days. In some of the fatalities, the erythrocyte count fell to 3,000,000 and less. No evidence of urolithiasis was observed in 30 rats (weighing approximately 200 g) which received 100 mg orally twice daily for 12 days, although 11 animals died within this time.

Sulfadiazine, on the contrary, produced renal failure in a number of infected mice. Since it was impossible to determine whether this was secondary to the damage caused by staphylococci, a series of normal mice and a group of normal rats were treated orally with similar and smaller doses of sulfadiazine. Gross inspection and microscopic examination (frozen sections of fresh tissue) of kidneys showed crystalline deposits, predominantly in the urinary papilla and often sufficiently massive to be capable of completely obstructing the urinary outflow. A more detailed study of this toxic manifestation will be reported elsewhere.<sup>22</sup>

*Conclusions.* 2-(Sulfanilamido)-5-ethyl-4-thiazolone (sulfaethylthiazolone) has less antistreptococcic activity and approximately the same antipneumococcic activity as sulfapyridine. Its antistaphylococcic activity is of the same order as that of sulfathiazole and sulfadiazine.

Sulfadiazine produces urolithiasis medicamentosa capable of causing death by acute suppression of urine in mice and rats, whereas sulfaethylthiazolone is free of this defect, but may cause fatal anemia.

## 13191

### Erythrocyte Phosphatase Activity in Hemolysed Sera and Estimation of Serum "Acid" Phosphatases.

ETHEL BENEDICT GUTMAN AND ALEXANDER B. GUTMAN.

*From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York City.*

Erythrocytes contain an apparently specific phosphatase<sup>1,2,3</sup> classified by Folley and Kay<sup>4</sup> as phosphomonoesterase A<sub>4</sub>. This enzyme

<sup>22</sup> Gross, P., Cooper, F. B., and Lewis, M., in press.

<sup>1</sup> Martland, M., Hansmann, F. S., and Robison, R., *Biochem. J.*, 1924, **18**, 1152.

<sup>2</sup> Roche, J., *Biochem. J.*, 1931, **25**, 1724.

<sup>3</sup> Roche, J., and Bullinger, E., *Enzymologia*, 1939, **7**, 278.

<sup>4</sup> Folley, S. J., and Kay, H. D., *Ergebn. d. Enzymforsch.*, 1936, **5**, 159.

in laked red cell systems readily dephosphorylates monophenylphosphate,<sup>2</sup> the pH range of activity for the reaction showing an optimum at 5.8-6.0 but extending to well below pH 5.0.<sup>2</sup>

In estimating the "acid" phosphatase activity of blood serum<sup>5, 6</sup> by a recently described adaptation<sup>7</sup> of the King and Armstrong "alkaline" phosphatase method,<sup>8</sup> monophenylphosphate substrate is used with citrate buffer at pH 4.9. These conditions also permit of hydrolysis by such erythrocyte phosphatase as may be present if sufficiently hemolysed samples of blood serum are employed. The determination of serum "acid" phosphatases (which has certain clinical applications<sup>9, 10, 11</sup>) therefore gives misleading results in markedly hemolysed blood samples. If the use of such samples is unavoidable, a correction can be made after hydrolysis in the presence of NaF, which in proper concentration differentially inhibits serum "acid" phosphatases.

*Experimental. Effect of hemolysis on phosphatase activity at pH 5.0.* Fresh, non-hemolysed, normal sera exhibit slight "acid" phosphatase activity: 1.4 and 1.6 units/100 cc respectively in the 2 subjects cited in Table I. Defibrinated whole blood samples from these normal subjects showed more than 100 times greater phosphatase activity when determined at the same pH (5.0) by the same method, because large amounts of erythrocyte phosphatase are present in the hemolysates. Fresh, non-hemolysed sera of patients with metastasizing prostatic carcinoma contain prostate "acid" phosphatase and show elevated "acid" phosphatase levels (Table I). Defibrinated whole blood samples from these patients exhibit further increases in phosphatase activity at pH 5.0, due to the added effect of erythrocyte phosphatase.

If blood samples are only partially hemolysed and less erythrocyte phosphatase is present in the serum, less distortion of "acid" phosphatase values occurs. When ordinary precautions against hemolysis are taken, normal sera (even if slightly tinged) show no significant erythrocyte phosphatase activity.<sup>5</sup> Collection of blood with a wet syringe, however, results in high values: 14.7 units/100 cc in one

<sup>5</sup> Gutman, A. B., and Gutman, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 470.

<sup>6</sup> Gutman, A. B., and Gutman, E. B., *J. Clin. Invest.*, 1938, **17**, 473.

<sup>7</sup> Gutman, E. B., and Gutman, A. B., *J. Biol. Chem.*, 1940, **136**, 201.

<sup>8</sup> King, E. J., and Armstrong, A. R., *Canad. M. A. J.*, 1934, **31**, 376.

<sup>9</sup> Robinson, J. N., Gutman, E. B., and Gutman, A. B., *J. Urology*, 1939, **42**, 602.

<sup>10</sup> Gutman, A. B., Gutman, E. B., and Robinson, J. N., *Am. J. Cancer*, 1940, **38**, 103.

<sup>11</sup> Woodard, H. Q., and Higinbotham, N. L., *J.A.M.A.*, 1941, **116**, 1621.

TABLE I.

Phosphatase Activity at pH 5.0 of Fresh, Non-hemolysed Serum and of the Corresponding Whole Blood Hemolysate; Effect of 0.005 *M* NaF. (0.005 *M* disodium phenylphosphate substrate, 0.1 *M* citrate buffer at 37°C. Results expressed in mg phenol liberated/100 cc/hr).

Source of blood	Serum	Serum + NaF	Inhibition by NaF %	Whole blood	Whole blood + NaF	Inhibition by NaF %
Normal subjects	1.4	0.1	93	210.	205.	2.4
	1.6	0.3	81	342.	338.	1.2
Cases of metasta-	56.0	7.2	87	259.	235.	9.3
sizing prostatic	10.8	2.6	76	60.*	57.*	5.0
carcinoma	60.0	7.2	88	172.**	169.**	1.7

\* Partial hemolysis.

\*\* This blood allowed to stand several days at room temperature. The prostate "acid" phosphatase has almost disappeared, the erythrocyte phosphatase present is little inhibited by NaF.

instance when the serum properly taken contained only 1.4 units. If such partially hemolysed samples of normal blood are allowed to stand several days, further increases occur. This does not apply to sera from patients with metastasizing prostatic carcinoma, as prostate "acid" phosphatase tends to disappear from the serum on prolonged standing at room temperature, in one case (after 72 hours) from 60.8 to 8.8 units/100 cc, despite some hemolysis.

*Influence of different substrates and buffers.* Monophenylphosphate appears to be the substrate of choice in the determination of serum "acid" phosphatase activity.<sup>7</sup> In hemolysed sera, however, the activity of erythrocyte phosphatase at pH 5.0 is more than 10 times greater with monophenylphosphate than with  $\beta$ -glycerophosphate substrate. As anticipated, marked distortion of serum "acid" phosphatase values was found also when  $\alpha$ -glycerophosphate substrate was used in hemolysed sera. Erythrocyte phosphatase activity is approximately 20% greater with acetate than with citrate buffer at pH 5.0.

*Effect of M/200 NaF.* Most of the "acid" phosphatase activity of normal sera, and of patients with metastasizing prostatic carcinoma is inhibited by incubation in the presence of M/200 NaF. Erythrocyte phosphatase, however, is not significantly inhibited. In fresh but hemolysed sera, the presence of appreciable amounts of prostate "acid" phosphatase is indicated by a significant fall in phosphatase activity after incubation with NaF; the difference approximates the prostate "acid" phosphatase present.

## Blood Pyruvic Acid in Heart Disease.

ZALE A. YANOF. (Introduced by E. S. Guzman Barron.)

*From the Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago, Chicago, Illinois.*

Meakins and Long<sup>1</sup> were the first to study intermediates of tissue carbohydrate metabolism in heart disease. They showed a marked and proportional increase in blood lactic acid in patients with heart failure. These results were subsequently both confirmed<sup>2</sup> and denied<sup>3</sup>. The importance of lactic acid as the center of carbohydrate metabolism has since been overshadowed by that of pyruvic acid, and more recently even more interest has attached to pyruvic acid because of Peters'<sup>4</sup> discovery that vitamin B<sub>1</sub> is necessary for its oxidation.

Taylor, S. Weiss, and Wilkins<sup>5</sup> attempted blood pyruvic acid estimations in all types of disease by measuring the blood bisulfite binding substances and showed a rise of these in failing hearts. This method of pyruvate estimation has since been shown, however, to be non-specific and inaccurate.<sup>6, 7</sup> This present work makes use of a specific method<sup>8</sup> and deals only with heart disease.

*Procedure.* The blood pyruvate levels of organically normal individuals and patients with decompensated and compensated heart disease were studied. The control group consisted of 5 male and 5 female carefully selected psychopaths, ranging in age from 25 to 84, non-alcoholics who were free of organic disease, had no evidence of dietary deficiency, and who were not excited or depressed. A pyruvate determination was done on each daily for 6 days and after one-half hour rest in bed. Twenty hospital patients, ages 32 to 79, with varying degrees of congestive failure, and with no fever, history of dietary deficiency, or impaired renal function other than that due to the cardiac failure, and no other organic disease, constituted the second group on whom daily blood pyruvate determinations were

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<sup>1</sup> Meakins, J., and Long, C. N. H., *J. Clin. Invest.*, 1927, **4**, 273.

<sup>2</sup> Harris, E. W., Jones, C. N., and Aldred, C. N., *Quart. J. Med.*, 1935, **4**, 407.

<sup>3</sup> Weiss, S., and Ellis, L. B., *Arch. Int. Med.*, 1935, **55**, 665.

<sup>4</sup> Peters, R. A., *Lancet*, 1936, **1**, 1161.

<sup>5</sup> Taylor, F. H. L., Weiss, S., and Wilkins, R. W., *J. Clin. Invest.*, 1937, **16**, 833.

<sup>6</sup> Wortis, H., Bueding, E., and Wilson, W. E., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 279.

<sup>7</sup> Elsom, K. O., Lukens, F. D. W., Montgomery, E. H., and Jones, L., *J. Clin. Invest.*, 1940, **19**, 153.

<sup>8</sup> Lu, G. D., *Biochem. J.*, 1939, **33**, 249.



TABLE I.\*  
Range and Mean Blood Pyruvate Levels of Controls.

Subject No.	24	25	26	27	28
Range**	.56-.80	.72-.84	.80-.98	.78-1.00	.80-.96
Mean	.68	.76	.92	.92	.88
Subject No.	29	30	31	33	34
Range**	.60-.88	.60-.90	.64-1.10	.60-.88	.64-1.00
Mean	.76	.79	.88	.73	.80

\*Values expressed here and elsewhere as mg per 100 cc of blood.

\*\*Range of each subject based on 6 daily determinations.

TABLE II.  
Decompensated Heart Group.

Subject No.	1	2	3	4	5	6	7	8	10	12
Etiology	H	H	A	A	A	A	A	H	A	H
Severity	S	S	S	M	M	M	S	M	S	S
Peak*	3.4	1.9	2.0	2.2	1.8	2.0	2.3	1.8	1.9	2.2
Subject No.	14	15	17	18	19	20	21	22	47	49
Etiology	A	R	R	U	H	R	H	CT	A	R
Severity	S	M	M	M	S	M	S	S	M	S
Peak	2.1	1.4	1.2	1.7	2.5	1.5	1.7	1.7	1.5	1.8

Abbreviations: H, hypertensive; A, arteriosclerotic; R, rheumatic; CT, coronary thrombosis; U, unknown; S, severe; M, moderate.

\*Highest blood pyruvate recorded.

made. In the third group were 12 patients, ages 20 to 62, with heart disease but without failure.

*Results and Discussion.* Table I reveals sharply defined limits of normality, with 1.0 as the upper limit and 0.8 as the mean.

The patients with decompensated hearts (Table II) show only one case, No. 17, whose highest blood pyruvate was not significantly elevated. The mean "peak" level for the group is 2.15, with a range of 1.2 to 3.4.\* Every case but one showed changes from day to day. These variations, however, were not considered significant unless they were higher than 0.3, the maximum day to day variation of the controls. An attempt was made to correlate the daily fluctuation with the clinical condition of the same day. Making allowance for one discordant variation, 79% of the patients show daily variations in agreement with the corresponding clinical changes. Or, of a sum total of 58 daily variations in all of the cases, 43 or 74% were clinically correlative.

It should be remembered, however, that clinical estimation of the change of a heart patient's condition from day to day is often diffi-

\* This roughly approximates the values that Lu<sup>9</sup> found in acute beriberi.<sup>9</sup> Platt, B. S., and Lu, G. D., *Quart. J. Med.*, 1936, **29**, 355.



TABLE III.  
Compensated Hearts.

Subject No.	35	36	37	38	39	41	43	44	53	54	55	56
Type	R	R	A	A	A	R	H	H	H	H	H	A
B. P.	0.80	0.56	0.80	0.56	0.72	0.80	0.88	0.80	0.64	0.72	1.16	0.84

Abbreviations: B. P., blood pyruvate; others as in Table II.

cult, if not at times impossible. It is quite possible, therefore, that even though the daily pyruvate level may not fit in with the clinical evaluation of that given day the former may still be the better index of the patient's true condition. That the mean peak of the severe failures was 2.13 as contrasted with 1.67 for those moderately ill, may be further evidence in this direction.

Of the 7 cases who died 3 showed a rise of pyruvate as death approached, 3 no change, and one a fall. It is interesting to note that 2 cases of edema of non-cardiac origin which were also studied showed no rise above the normal at any time. The age, sex, and type of heart disease could not be shown to bear any relationship to the pyruvate level.

For the compensated group (Table III) 12 patients with definite cardiac enlargement (10 to 110% oversized) were selected for study. The pyruvate levels were well within normal limits except for one, No. 55, who showed a value of 1.16. Of further interest were 2 ambulant patients with heart disease who appeared to be compensated except for a definite but slight edema, and they both had increased values of 1.2.

*Summary.* 1. There is a rise above the normal of blood pyruvic acid in heart failure. 2. This elevation approximates the degree of failure.

The author is very grateful to Dr. Emmet B. Bay for his helpful suggestions and criticism and to Dr. E. S. G. Barron for the use of his laboratory and his technical guidance.

## 13193

## Effect of Neural Stimulation on Choline-Esterase Activity of the Blood.

CAROLYN TROWBRIDGE. (Introduced by W. O. Fenn.)

*From the Department of Physiology, School of Medicine and Dentistry, The University of Rochester, Rochester, N. Y.*

Croxatto, Huidobro, Croxatto, and Salvestrini<sup>1</sup> published a paper showing an increase of almost 100% in the choline-esterase activity of the renal blood of the cat, during neural stimulation of the leg muscles. In an attempt to verify these findings, similar experiments were performed on the frog as well as on the cat with modified technics.

For the frog experiments, a perfusion technic was used. The cannulae were introduced into the dorsal aorta and the ventral abdominal vein as described by Fenn, *et al.*<sup>2</sup> The perfusion fluid was a mixture of gum acacia, Ringer's solution, and beef red blood cells.<sup>3</sup> Five experiments were done, analyzing only the supernatant fluid after the red cells had been removed by centrifugation, and one experiment in which the whole perfusion fluid was analyzed. In all cases, analyses were made of arterial samples and of venous samples taken, (a) in the resting state, (b) during about 10 minutes of electrical stimulation of the leg muscles through the sciatic nerve, and, (c) after stimulation had ceased. The stimulation was accomplished by means of a condenser discharge type stimulator. The frequency and intensity of the shocks were adjusted to produce maximal contraction and were not constant. The samples were analyzed for choline-esterase with the differential volumeter apparatus of Fenn<sup>4</sup> according to the principle used by Stedman and Stedman.<sup>5</sup> One cc of bicarbonate buffer (.65% NaCl and .4% NaHCO<sub>3</sub>), and one-half cc of 1% acetylcholine solution in saline were placed in the bottom of the flask, and 1/32 to 1/4 cc of the test solution, depending on the activity, was placed in the side arm. In cases where there was appreciable hemolysis of the red blood cells, the degree of hemolysis

<sup>1</sup> Croxatto, H., Huidobro, F., Croxatto, R., and Salvestrini, H., *Comptes Rendus Soc. Biol.*, 1939, **130**, 236.

<sup>2</sup> Fenn, W. O., Koenemann, R. H., and Sheridan, E. T., *J. Cell. and Comp. Physiol.*, 1940, **16**, 255.

<sup>3</sup> Saslow, G., *Am. J. Physiol.*, 1938, **124**, 360.

<sup>4</sup> Fenn, W. O., "The Differential Volumeter for the Measurement of Cell Respiration and Other Processes." *The Science Press*, 1935.

<sup>5</sup> Stedman, E., and Stedman, E., *Bioch. J.*, 1935, **292**, 2107.

was determined by measuring the hemoglobin content of the supernatant fluid by dilution and color comparison. In the experiment using the whole perfusion fluid, the amount of dilution was calculated from a measure of the hemoglobin content by the acid hematin method.<sup>6</sup>

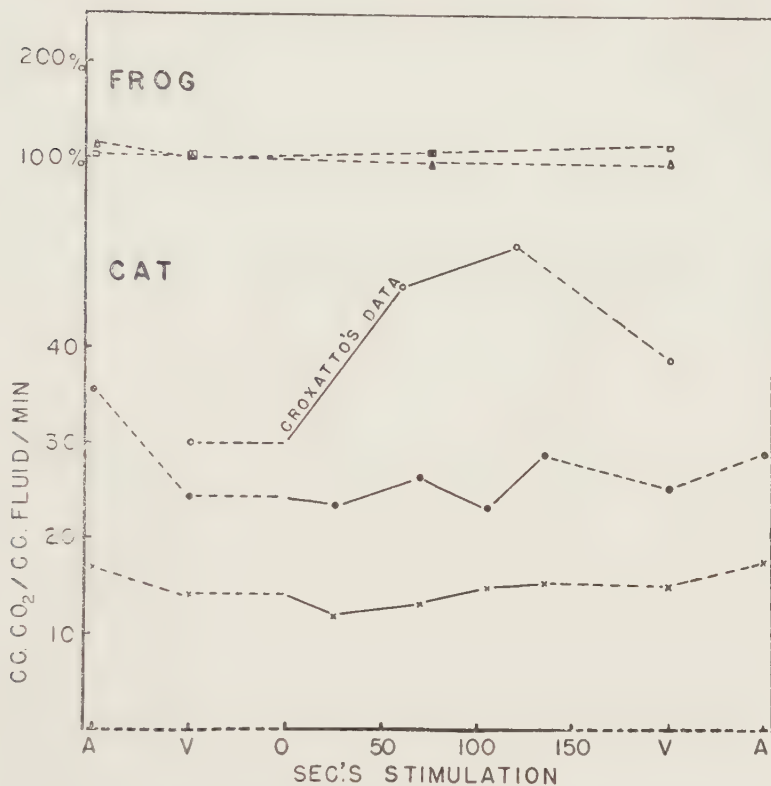
Two experiments were done on the cat. The animals were anesthetized with Dial and injected with chlorazol fast pink (1 cc of 5% solution/kg) to prevent blood clotting. Blood samples were taken by cannula from the saphenous vein<sup>7</sup> before stimulation, during 2 minutes of stimulation of the leg muscles through electrical excitation of the severed sciatic nerve in the sciatic notch, and 10 minutes after stimulation had ceased. Other samples were taken from the carotid artery at the beginning and end of the experiments. Both whole blood and plasma of each sample were analyzed for choline-esterase by the same method as in the frog experiments. In both experiments, records of the tension developed by the gastrocnemius group of one leg were made and found to show maximal response. On each of these blood samples, plasma potassium analyses had been made by Mr. J. R. Jordan in connection with another experiment and these determinations showed a normal rise of potassium on stimulation, indicating normal functioning of both muscle and nerve.

The results of the frog experiments were negative. When there was no hemolysis of the red cells, there was little or no choline-esterase activity in the plasma and the amount bore no relation to stimulation. When the red cells had hemolysed, the activity was proportional to the degree of hemolysis, *i. e.*, plotting the activity against the percent of hemolysis gave a straight line. In the experiment in which the whole perfusate was analyzed, the data showed no correlation between stimulation and high choline-esterase activity of the perfusate.

In the cat experiments, the results were quite different from those of Croxatto *et al.*<sup>1</sup> In these experiments there was a rather wide scattering of the values found but again there was no correlation with stimulation. A summary of the frog data and the data on Cat I are shown on the graph (Fig. 1). The second cat experiment was not as complete as No. 1 (fewer samples were taken) but the choline-esterase value for the one stimulated sample was not statistically different from those of the resting and arterial samples. The average

<sup>6</sup> Hawk, P. B., and Bergeim, O., "Practical Physiological Chemistry," Blakiston, 11th edition.

<sup>7</sup> Fenn, W. O., Wilde, W. S., Boak, R. A., and Koenemann, R. H., *Am. J. Physiol.*, 1939, **128**, 39.



For the 5 frog experiments the points are the average choline-esterase activities expressed as percent of the resting venous value. The solid symbols indicate stimulation.  $\square$  = whole perfusate;  $\triangle$  = supernatant fluid (the average of four experiments). The lower curves show the results of the first cat experiment.  $\bullet$  = whole blood;  $\times$  = plasma. The cat plasma values are computed by averaging the results of direct measurement and values obtained by back calculation from the whole blood values and the hematocrit. An average value for choline-esterase content of the red blood cells was computed and subtracted from the whole blood value for each sample in proportion to the hematocrit of that sample. The figures of Croxatto *et al.* are included for comparison. The vertical scale is arbitrary. In all curves, the solid portions of the line are plotted using seconds of stimulation as the abscissa; the dotted portions have no time scale except that the readings were made before and after stimulation as indicated. A is arterial and V is non-stimulated venous.

values were: stimulated venous, 16.5 cc CO<sub>2</sub>/cc fluid/min; resting venous, 16.7; arterial, 15.6. In the first cat experiment the values were: stimulated venous, 25.5; resting venous, 24.4; arterial, 32.2.

These experiments indicate that stimulation of the leg muscles in either the frog or the cat does not cause an increase in the choline-esterase activity of the venous blood coming from that limb. The discrepancy between these results and those of other authors may be

due to the fact that, in the work presented here, the blood was taken directly from the limb, the saphenous vein of the cat, while in the previous work, the renal vein was the source of the blood samples. The increase of choline-esterase found under those circumstances may be due to some other factor in some other part of the body rather than to a direct output of choline-esterase by the stimulated muscles.

I wish to acknowledge my indebtedness to Dr. W. O. Fenn and Dr. J. C. Sabine for their assistance in both technical problems and interpretation of results.

### 13194

#### Action of Nicotinic Acid on Coagulation of the Blood.

P. M. AGGELER AND S. P. LUCIA.<sup>†</sup> (With the technical assistance of Alexis Astaff.)

*From the Divisions of Medicine and Preventive Medicine, University of California Medical School, San Francisco.*

Calder and Kerby<sup>1</sup> recently reported that the prolonged coagulation time of heparinized blood could be significantly shortened *in vitro* by the addition of 1% nicotinic acid. They stated "... that nicotinic acid does not duplicate the action of, nor can it be substituted for, any of the known factors involved in blood clotting," and furthermore suggested that its effect might be due to chemical neutralization of antithrombin.

The following experiments were performed in order to investigate the mechanism of action of nicotinic acid on the coagulation of the blood.

*Methods.* The coagulative potency of 1% nicotinic acid in 0.85% NaCl; 1% nicotinic acid amide in 0.85% NaCl; Nicamin,\* representing 1% nicotinic acid; 0.001% protamine;\*\* thromboplastin solution;<sup>2</sup> 0.85% NaCl; and distilled water was tested with the blood of 4 healthy human adults in whom the bleeding time, coagu-

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<sup>†</sup> Assisted by grants from Mr. Frank Kennedy and the Pacific Institute of Tropical Medicine.

<sup>1</sup> Calder, R. M., and Kerby, G. P., *Am. J. Med. Sci.*, 1940, **200**, 590.

\* Monoethynolamine nicotinate, Abbott. Dilutions of the commercial product representing 25 mg of nicotinic acid per cc were made with 0.85% NaCl.

\*\* Kindly supplied by Eli Lilly & Co.

<sup>2</sup> Quick, A. J., *J.A.M.A.*, 1938, **110**, 1658.



lation time, blood clot retraction, prothrombin concentration, and platelet count was normal. All tests were done in a water bath at 37°C and were timed with a stopwatch.

*Plasma Experiments:* These tests were performed in triplicate by methods similar to those previously reported.<sup>3</sup> Glass test tubes were used (10 x 75 mm), 9 cc of blood was obtained by venipuncture, immediately mixed with 1 cc of 1.34% sodium oxalate solution and centrifugalized at 2000 r.p.m. for 30 minutes. 0.1 cc of a 0.85% NaCl solution containing 2 units of heparin<sup>†</sup> was added to each cc of the supernatant plasma. 0.1 cc heparinized plasma was mixed with 0.1 cc of each of the substances to be tested. 0.1 cc of a 0.27% CaCl<sub>2</sub> solution was added to each mixture and the coagulation time noted. Control tests with 0.85% NaCl and thromboplastin solutions were done with unaltered plasma and whole blood.

*Whole Blood Experiments:* These tests were performed in duplicate. Glass test tubes (13 x 100 mm) were used, 0.2 cc of a 0.85% NaCl solution containing 0.75 units of heparin<sup>†</sup> per cc was mixed with 0.9 cc of each of the test substances. 1.5 cc of whole blood obtained by venipuncture was immediately added to each tube and the coagulation time noted.

*Hemolytic Tests.* 1.5 cc of a 2% suspension of washed sheep cells was added to 1.5 cc of each test substance and observed for hemolysis, immediately and at intervals up to 1½ hours.

*Results.* The results of the plasma experiments are given in Table I. The coagulation time remained prolonged beyond 1800 seconds in all samples to which nicotinic acid, nicotinic acid amide, Nicamin, 0.85% NaCl, or distilled water had been added. The average coagulation time was shortened to 238 seconds in the samples to which protamine was added, and to 36 seconds in the samples to which thromboplastin was added. In the control samples the average coagulation times were 173 and 15½ seconds. In a separate series of experiments it was determined that 0.001% protamine alone is slightly anti-coagulant when tested with plasma.

It is apparent that nicotinic acid, nicotinic acid amide and Nicamin are ineffectual when tested with heparinized plasma.

The results of the whole blood experiments are given in Table II. The coagulation time remained prolonged beyond 3600 seconds in the samples to which nicotinic acid amide, Nicamin, or 0.85% NaCl were added. It was shortened to an average of 1037 seconds in the

<sup>3</sup> Aggeler, P. M., and Lucia, S. P., *Am. J. Med. Sci.*, 1940, **199**, 181.

<sup>†</sup> Connaught Laboratories—1000 units per cc.

TABLE I.  
Effect of Various Test Substances on Coagulation of Human Plasma.

		Coagulation Time in Seconds				
		Subj. A	Subj. B	Subj. C	Subj. D	Average
Heparinized Plasma	+	0.85% NaCl	>1800	>1800	>1800	>1800
		Distilled water	>1800	>1800	>1800	>1800
		Nicotinic acid	>1800	>1800	>1800	>1800
		“ “ amide	>1800	>1800	>1800	>1800
		Nicamin	>1800	>1800	>1800	>1800
		Protamine	238	236	238	240
Unaltered Plasma	+	Thromboplastin	43	32	34	35
		0.85% NaCl	215	163	166	150
		Thromboplastin	15	15	16	16

samples to which nicotinic acid was added, to 1144 seconds in distilled water, to 349 seconds in protamine, and to 29 seconds in the samples to which thromboplastin was added. In the control samples the average coagulation times were 243 and 10 seconds.

It is apparent that the limited effect of nicotinic acid is similar to that of distilled water and that the two compounds of nicotinic acid are without significant effect.

In order to further elucidate the problem, it seemed advisable to investigate the hemolytic effect of the test substances. The results of the tests are given in Table III. Distilled water induced immediate and complete hemolysis. Following an initial delay of several minutes, the nicotinic acid appeared to promote complete hemolysis with the production of a brownish tinged fluid, characteristic of acid hematin. Within 1½ hours a flocculant mass of laked red blood cells and cellular debris had settled to the bottom of the tube.

From these experiments it appears that nicotinic acid induces coagulation of heparinized blood by damaging the formed elements contained therein. Such action would release thromboplastin and

TABLE II.  
Effect of Various Test Substances on Coagulation of Human Blood.

		Coagulation Time in Seconds				
		Subj. A	Subj. B	Subj. C	Subj. D	Average
Heparinized Whole Blood	+	0.85% NaCl	>3600	>3600	>3600	>3600
		Distilled water	1230	885	1560	900
		Nicotinic acid	967	1080	1100	990
		“ “ amide	>3600	>3600	>3600	>3600
		Nicamin	>3600	>3600	>3600	>3600
		Protamine	330	347	345	375
Whole Blood	+	Thromboplastin	40	38	18	20
		0.85% NaCl	200	247	285	240
		Thromboplastin	10	9	10	11

TABLE III.  
Comparative Hemolytic Effect of Test Substances on Sheep Erythrocytes.

Substance	Degree of Hemolysis
0.85% NaCl	0
Distilled water	++++
Nicotinic acid	++++
“ “ amide	+
Nicamin	+
Protamine	0
Thromboplastin	0
Heparin solution	0

thus accelerate the conversion of prothrombin to thrombin, thereby overcoming the anticoagulant action of heparin.<sup>4</sup>

*Summary.* Nicotinic acid has no coagulant effect when tested *in vitro* with heparinized recalcified plasma. Its coagulant action is comparable to that of distilled water when tested *in vitro* with heparinized whole blood. Nicotinic acid is an active hemolytic agent and its coagulant effect is apparently due to the release of thromboplastin from the disrupted elements of the blood. Compounds of nicotinic acid which do not produce significant hemolysis have no appreciable coagulative potency.

### 13195 P

#### Effect of Milk on Gizzard Erosion and Cholic Acid in the Chick.

H. J. ALMQUIST, E. MECCHI AND F. H. KRATZER.

*From the Division of Poultry Husbandry, College of Agriculture, University of California, Berkeley.*

The preventive and curative effect of cholic acid on dietary gizzard erosion of chicks has been reported.<sup>1, 2</sup> The effect is similar in the case of gizzard erosions produced by the use of either a basal diet or a practical rearing diet to which cincophen has been added.<sup>2</sup> We desire to report at this time evidence for the existence in cow's milk of a labile substance which acts like cholic acid.

Dried milk products were fed by mixing in the diets; liquid milk products were given to the chicks in place of the drinking water.

<sup>4</sup> Quiek, A. J., *Am. J. Physiol.*, 1936, **115**, 317.

<sup>1</sup> Almquist, H. J., and Mecchi, E., *J. Biol. Chem.*, 1938, **126**, 407.

<sup>2</sup> Almquist, H. J., and Mecchi, E., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 168.

TABLE I.  
Effect of Milk Products on Cholic Acid Content and Gizzard Erosions in Chicks.

Supplement to basal diet	Level %	Gallbladder bile			
		Volume per chick cc	Cholic acid		Gizzard erosion score
			Per gm dried bile mg	Per chick mg	
None	10	.16	61	3	1.04
Dried whole milk		.18	86	4	1.10
Whole milk, raw		.28	135	11	.50
“ “ boiled		.28	131	10	.30
Skim milk, raw		.31	134	11	.42
Supplement to practical mash					
None		.25	169	11	.06
Skim milk, raw		.55	316	39	.00
Cincophen	1	.23	206	13	.78
“ “	1	.50	368	39	.09
+ skim milk, raw					

The diets and the details of the experimental procedure have been described.<sup>1, 2</sup>

The results given in the table show no effect on the characteristics measured in the case of dried whole milk. This has also been found true in the case of commercial dried skim milk, dried buttermilk and dried whey. On the other hand, liquid milk products distinctly reduce the severity of gizzard erosion, increase the gall bladder bile volume per chick and increase the quantity of cholic acid per chick. The data show that this effect of liquid milk products is stable to heating up to the boiling point. Other experiments have indicated that the activity is not removed by steam distillation. The effects of liquid milk products are very distinct with both types of erosion producing diets.

The evidence presented is consistent with earlier reports<sup>1</sup> linking gizzard erosions with a deficient formation of bile, especially of cholic acid, within the chick. It is further indicated that a substance with an activity like that of cholic acid exists in cow's milk. Attempts to detect cholic acid in skim milk yielded only negative results.

## High Frequency Conductivity and Dielectric Effect of Fresh Fertile and Infertile Hens' Eggs.

ALEXIS L. ROMANOFF AND KARL FRANK.

*From the Department of Poultry Husbandry, Cornell University Agricultural Experiment Station, Ithaca, New York.*

In the previous studies of Romanoff and Cottrell<sup>1</sup> evidence was presented that at radio frequencies from 14 to 14.4 megacycles the conductivity is lower and the dielectric effect higher in fresh fertile hens' eggs than in infertile. In view of these results it was deemed desirable to extend the study of the intact egg to higher frequencies. Also to follow up this work an investigation of the physical properties of each component of the egg—yolk and various layers of albumen—was required in order to determine the exact seat of the differences between fertile and infertile eggs and the conditions under which they are maximum.

*Methods and materials.* To cover the range between 2 and 60 megacycles, 2 vacuum tube oscillators were used. The source for the lower frequency range, from 2 to 15 megacycles, was the oscillator described in the previous article.<sup>1</sup> For the generation of the higher frequencies, 15 to 60 megacycles, the special oscillator was assembled (Fig. 1-A). The power supplies for both oscillators were fed from a 60 watt voltage regulator and there was negligible frequency drift or detuning on loading.

The oscillators were coupled inductively to a resonant tank circuit which was connected in parallel with a pair of circular plates forming the dielectric cell. The 3 to 15  $\mu\mu\text{f}$  variable tank condenser was provided with a very accurate micrometer worm drive and dial permitting settings of the condenser capacity to an accuracy of  $\pm 0.002 \mu\mu\text{f}$ . In the center of the tank coil was mounted a small 2.3 v flashlight bulb above which was a photronic cell protected by a copper sulphate heat filter. A galvanometer shunted to critical damping registered the intensity of the light from the bulb.

The entire apparatus was placed in a shielded cage and operated by remote control. The power output from the oscillators showed a long period drift of less than 1 cm galvanometer scale reading per hour for the ultra-high frequency oscillator and somewhat more for the lower frequency oscillator.

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<sup>1</sup> Romanoff, A. L., and Cottrell, C. L., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **42**, 298.



When measurements on whole eggs were being made the egg was mounted inside the resonant tank coil by means of an egg-holder sliding on a track and consisting of 2 opposing glass cups pulled together by a spring. The long axis of the egg was thus automatically made to coincide with the principal axis of the coil. By this method the effect of position of the egg was reduced to a minimum and readings on an individual egg could be repeated very precisely. The coupling between the oscillator and the resonant tank was standardized by means of a glass egg filled with egg albumen.

In studying the separate components of the egg, 10 g samples were weighed out to 0.01 g in small Petri dishes, 50 mm in diameter, and introduced between the plates of the dielectric cell. The readings on yolk and middle dense albumen were made alternately on fertile and infertile individual eggs. Outer and middle fluid layers of albumen, owing to the small amounts available from each egg, were studied from composite samples only.

The fertile and infertile eggs in these investigations were obtained from 2 biologically identical flocks of White Leghorn hens (*Gallus domesticus*). The eggs from the flock with males were tested for fertility either by incubation or by examination of the blastoderm in dissected eggs.

*Experimental.* Measurements of conductivity and dielectric effects on several lots of intact eggs at frequencies of 54.6 and 27.3

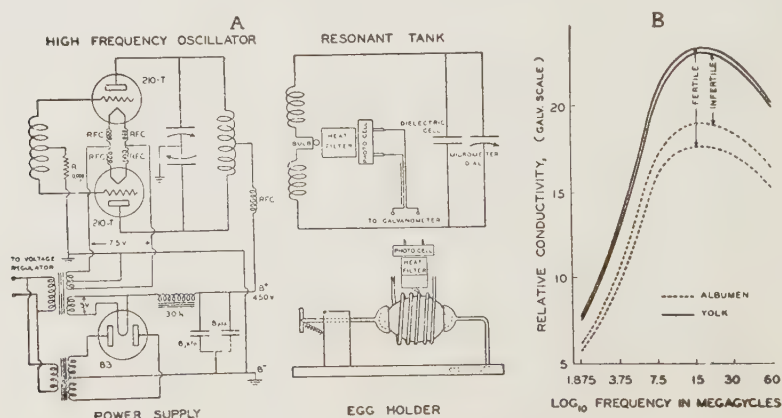


FIG. 1.

A. Circuits of power supply, high frequency oscillator and resonant tank. The 450 volt power supply feeds a tuned plate-tuned grid type oscillator using two 210-T's in push-pull mounted base to base, which in turn drives the resonant tank with egg coil or dielectric cell.

B. The relative conductivity measurements on yolk and dense albumen of fresh fertile and infertile eggs plotted against the logarithm of the frequency in megacycles. (Based on the observations of 20 dissected eggs in equal numbers of fertile and infertile).

megacycles showed tendencies in relative values of fertile and infertile eggs very similar to those previously observed at lower frequencies.<sup>1</sup> There was greater separation in conductivity in favor of infertile eggs. The dielectric effect was less consistent, although again showing higher values for fertile than for infertile eggs.

The measurements of the conductivity and dielectric effect of the egg components yolk and albumen were then made to locate the region where the principal differences between fertile and infertile eggs occur. The curves on relative conductivity (Fig. 1-B) show that the values for various parts of the egg were maximum at about 15 megacycles. The difference in power absorption of any sample between high and low frequencies was large, but the percentage difference between 2 different samples remained almost constant over the entire frequency range employed. The observations on the dielectric effect show that there was little increase in values and a gradual levelling off toward lower frequency.

The separation in values of relative conductivity of yolk and albumen of infertile eggs was only 18.6%, while of fertile eggs it increased to 28.4%. It should be noted that with fertilization the conductivity for the yolk was greater, while that for albumen was smaller, thus tending to mask the detection of fertility of the intact egg. For the dielectric effect the reverse is true. The separation of yolk and albumen was decreased from 13.2% in the infertile egg to 10.6% in the fertile egg.

Since several distinct layers of albumen are known<sup>2</sup> the relative values for conductivity and dielectric effect of the 3 outermost layers—outer liquid, middle dense, and middle liquid—were determined (Table I). The measurements of the outer fluid gave

TABLE I.  
Effect of Fertility on Relative High Frequency Conductivity and Dielectric  
Effect of Various Egg Components.<sup>1</sup>  
(at F = 15 megacycles)

Egg components	Conductivity			Dielectric Effect		
	galv. scale readings			dial scale readings		
	Infertile	Fertile	% diff.	Infertile	Fertile	% diff.
Layers of albumen:						
Outer liquid	16.15	14.10	— 14.5	78.15	76.05	— 2.1
Middle dense	17.03	15.90	— 13.4	77.40	76.43	— 1.3
Middle liquid	12.35	11.15	— 10.8	74.10	71.70	— 3.4
Yolk	20.20	20.42	+ 1.1	69.22	69.13	— 0.13

<sup>1</sup> Based on the observations of 28 dissected eggs in equal numbers of fertile and infertile.

<sup>2</sup> Romanoff, A. L., and Sullivan, R. A., *Ind. and Eng. Chem.*, 1937, **29**, 117.

substantially the same values as the middle dense layer for conductivity and dielectric effect. On the other hand, the middle fluid showed a considerably lower value of conductivity than any of the other parts of the egg, while its dielectric effect lay about midway between those of the yolk and middle dense albumen.

These data indicate that the difference between the fertile and infertile egg in relation to conductivity and dielectric effect occurs principally in the albumen. The conductivity of fertile eggs was 7.4% lower than that of infertile eggs. The dielectric effect showed tendencies in favor of fertile eggs, but the differences were exceedingly small and of the same order of magnitude as the probable error. The values for the yolk showed no appreciable difference between fertile and infertile eggs.

*Summary.* The apparatus and methods for the precise measurements of small differences in the high frequency conductivity and dielectric effect of the intact egg and samples of the contents of the egg have been described.

The relative conductivity and dielectric effect of the intact egg at frequencies of 27.3 and 54.6 megacycles gave results indicating that fresh infertile eggs have a higher conductivity and a tendency towards lower dielectric constant than fertile eggs.

In dissected eggs the difference between the fertile and infertile egg in relation to conductivity and dielectric effect was observed to occur principally in the albumen. The conductivity of the albumen of fertile eggs was 7.4% lower than that of infertile eggs.

The conductivity values for various parts of the egg were maximum at about 15 megacycles. There was no frequency dependence for the percentage separation between yolk and albumen over the range from 2 to 60 megacycles.

## 13197 P

## An Improved Capillary Microrespirometer.

JULIAN TOBIAS AND R. W. GERARD.

*From the Department of Physiology, University of Chicago.*

Instruments for the measurement of tissue and cell respiration have steadily moved, since the introduction of Warburg's convenient manometer, in the direction of smaller volume and greater sensitivity. Gerard and Hartline<sup>1</sup> took advantage of the greater stability afforded by reducing the tissue chamber of a volumeter to capillary dimensions (0.5 to 1.2 mm diameter) and having this inside the relatively large "differential" chamber. Index drop movements, followed with an ocular micrometer, were consistent over 5-minute intervals, even when corresponding to volume changes of about 0.01 cmm. The diver technique, introduced by Linderström-Lang,<sup>2,3</sup> is of the same order of sensitivity and consistency; and the electrical method<sup>4</sup> gives promise of superior performance.

We have further developed the capillary method so that it is convenient to follow the respiration of 10 tissue samples at once, and it is possible to measure absolute gas volume changes of 0.001 cmm, minute by minute, with an error of some 8% minute by minute, under 1% for longer intervals. The respiration even of bits of frog sciatic nerve weighing a fraction of a milligram can thus be followed at half-minute intervals.

The tissue chamber, also containing filter paper bits soaked with acid or alkali and separated from each other and the tissue by dry paper guards, is a short length of capillary of 1.2-1.5 mm internal diameter. The "open" end of this, after the insertion of materials, is plugged with plasticine. Into the other end has been cemented a fine drawn capillary of about 0.2 mm diameter. At the close of the experiment, this capillary is broken off and its end diameters accurately measured by end on examination with an ocular micrometer. Several such units are mounted on a central rod extending from a glass stopper, which ultimately fits into a glass container to provide a completely closed system. This is then immersed in a thermostat with the capillaries horizontal, and the whole is rotated about its long axis so that one or another capillary is brought into

<sup>1</sup> Gerard, R. W., and Hartline, H. K., *J. Cell. and Comp. Physiol.*, 1934, **4**, 141.

<sup>2</sup> Linderström-Lang, K. L., *Nature*, 1937, **140**, 108.

<sup>3</sup> Boell, E. J., Needham, J., and Rogers, V., *Proc. Roy. Soc., Series B*, 1939, **127**, 322.

<sup>4</sup> Davies, P. W., and Brink, F., *Proc. Am. Physiol. Soc.*, 1941, 69.

the field of a long focus horizontal microscope. A droplet of synthetic isodecane (2,7 dimethyl octane kindly given us by Dr. Goldsby) is placed at the open end of each small capillary before the system is closed. While the system is reaching temperature equilibrium the droplets are drawn well in from the tube tip, where they otherwise tend to be held.

With such fine capillaries the usual index fluids—purified kerosene, mineral oil, xylol, and Ringer solution—proved unsatisfactory, although with capillaries of about 1 mm diameter Ringer solution serves well. (Incidentally, several samples of kerosene showed considerable evaporation and attendant pressure changes.) To control possible constant errors, an absolute calibration of amount and speed of response was made by altering the pressure in the outside chamber in a known manner. The drop movement was found to measure the theoretical volume change within the accuracy with which this latter was known (5%); and to reach its final position, even after large movements, within a few seconds.

In each experiment, capillaries, with and without reagents, served as thermometric controls; but their droplets rarely moved after the initial attainment of temperature equilibrium. The other tubes, containing tissue, manifested a steady and smooth oxygen consumption which usually fell slowly and continuously with time. Over time intervals of 10 minutes or more, the accuracy of readings was better than 1%; and variations in  $QO_2$  from one tissue sample to another are attributable to differences in the samples. Actually the reliability of  $QO_2$  values is limited by the accuracy of estimation of

TABLE I.

Tissue	Amount	Micrometer divisions moved in successive intervals (1 div. = 0.004 mm)	$QO_2$	
			90 min.	150 min.
Whole blood	4. cmm	7.8, 7.9, 7.1, 5.5, 7.2 (60 sec. inter.)	30	
Sciatic	0.62 mg	3.6, 2.4, 2.3, 2.6 (60 sec.)	70	
Brain	0.69 "	11.0, 13.0, 12.0, 13.0, 15.6, 14.4, (30 sec.)	442	
No. exp. averaged			90 min.	150 min.
Primordium				
Pall	16		530	449
Cerebellum	11		462	359
Ant. Olfactory	8		440	352
Nuc.				
Ventral Off.	6		457	378
Bulb				



the amount of respiring material. Weighing a half milligram of tissue, even on a semi-micro balance, permits a considerable percentage error; but forcing the tissue into a capillary and measuring its volume gives promise of greater accuracy. That the injury attendant upon the initial dissection is not a constant source of error is shown by the essentially similar values obtained from samples of large and small fragments of the same type of tissue. Such tests likewise indicate an adequate gas diffusion and absorption.

Some actual respiration values obtained on frog blood and nervous tissue, and the reliability of individual readings, are indicated in the tables.

13198

#### Anti-sulfapyridine and Anti-sulfathiazole Effect of Local Anaesthetics Derived from p-Aminobenzoic Acid.

A. K. KELTCH, LINVILLE A. BAKER, M. E. KRAHL, AND  
G. H. A. CLOWES.

*From the Lilly Research Laboratories, Indianapolis, Indiana.*

It was shown by Woods<sup>1</sup> that the bacteriostatic effect of sulfanilamide or sulfapyridine on hemolytic streptococci *in vitro* could be blocked by small concentrations of p-aminobenzoic acid. Also the diethylaminoethyl ester of p-aminobenzoic acid, used as a local anaesthetic under the names 'Novocaine' or 'Procaine,' was almost as strongly anti-bacteriostatic as p-aminobenzoic acid itself; in concentrations of  $5.8 \times 10^{-8}M$  these compounds could suppress the effect of  $3.03 \times 10^{-4}M$  sulfanilamide.

Owing to the increasing local and systemic use of sulfonamides in treatment of wounds and in surgery where local anaesthetics are concurrently employed, it appeared of interest to determine first, whether or not the anti-bacteriostatic effect of 'Novocaine' could be obtained with other local anaesthetics and, secondly, whether or not local anaesthetics which were not p-aminobenzoic acid derivatives would exhibit an anti-bacteriostatic effect similar to that of 'Novocaine.' To this end, 7 local anaesthetics derived from p-aminobenzoic acid and 9 local anaesthetics not derived from p-aminobenzoic acid have been tested *in vitro* for their ability to

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<sup>1</sup> Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

TABLE I.

Effect of 7 Local Anesthetics Derived from p-Aminobenzoic Acid (Nos. 2-8) and 9 Local Anesthetics not Derived from p-Aminobenzoic Acid (Nos. 9-17) on Bacteriostasis of *B. coli* by Sulfapyridine. The Bacterial Population after 24 hr of Growth is Expressed in Terms of Photoelectric Density Readings; a Reading of 100 Represents Approximately 1 Billion Organisms per cc; a Reading of 300 Represents About 6 Billion Organisms per cc.

Local Anesthetic	Conc. local anesthetic	Growth in various molar conc. of Sulfapyridine			
		0	$2 \times 10^{-4}$	$8 \times 10^{-4}$	$3.2 \times 10^{-3}$
	moles per l				
1. p-Aminobenzoic Acid (Not an anesthetic— used as control)	0	356	114	109	83
	$10^{-6}$		374	159	91
	$10^{-5}$		351	337	305
	$10^{-4}$	375		348	308
2. $\beta$ -Diethylaminoethyl-p-amino- benzoate 'Novocaine'	0	356	114	109	83
	$10^{-5}$		251	96	90
	$10^{-4}$		348	229	109
	$10^{-3}$	383	372	359	303
3. $\gamma$ -Dimethylamino- $\alpha$ , $\beta$ -dimethylpropyl- p-aminobenzoate	0	356	114	109	83
	$10^{-5}$		364	133	92
	$10^{-4}$		355	350	261
	$10^{-3}$	362	338	335	343
4. $\gamma$ -Diethylamino- $\beta$ , $\beta$ -dimethylpropyl- p-aminobenzoate	0	356	114	109	83
	$10^{-5}$		113	87	80
	$10^{-4}$		279	94	78
	$10^{-3}$	362	341	268	119
5. $\beta$ -Diethylaminoisohexyl-p-amino- benzoate	0	378	107		
	$10^{-6}$		101		
	$10^{-5}$		175		
	$10^{-4}$		334		
	$10^{-3}$	359	353		
6. $\gamma$ -Dibutylaminopropyl-p-amino- benzoate	0	378	107		
	$10^{-5}$		106		
	$10^{-4}$		172		
	$10^{-3}$	353	326		
7. $\beta$ -Dimethylaminoethyl-p- butylaminobenzoate	0	378	107		
	$10^{-4}$		100		
	$10^{-3}$	355	176		
8. Ethyl-p-aminobenzoate	0	360	103		
	$10^{-4}$		106		
	$10^{-3}$	328	147		
9. $\gamma$ -(2-Methyl-piperidino)-propyl- benzoate	0	356	114	109	83
	$10^{-5}$		106	86	81
	$10^{-4}$		106	81	83
	$10^{-3}$	359	136	83	80
10. $\gamma$ -Diethylaminopropyl cinnamate	0	356	114	109	83
	$10^{-5}$		139	88	75
	$10^{-4}$		115	84	82
	$10^{-3}$	340	117	74	62

TABLE I. (Continued).

Local Anesthetic	Conc. local anesthetic moles per l	Growth in various molar conc. of Sulfapyridine			
		0	$2 \times 10^{-4}$	$8 \times 10^{-4}$	$3.2 \times 10^{-3}$
11. 2-Butyloxyquinoline carboxylic acid-4-diethylethylenediamide	0	356	114	109	83
	$10^{-5}$		100	96	78
	$10^{-4}$		113	80	78
	$10^{-3}$	358	106	17	22
12. Piperidinopropanediol-di-phenylurethane	0	360	103		
	$10^{-6}$		94		
	$10^{-5}$		106		
	$10^{-4}$		108		
13. Ethenyl-p-diethoxydiphenylamidine	0	378	107		
	$10^{-5}$		93		
	$10^{-4}$		105		
	$10^{-3}$	383	68		
14. 4-Benzoxo-2,2,6-trimethylpiperidine	0	378	107		
	$10^{-5}$		100		
	$10^{-4}$		101		
	$10^{-3}$	357	102		
15. 2-Benzoxo-2-dimethylamino-methyl-1-dimethylaminobutane	0	360	103		
	$10^{-5}$		114		
	$10^{-4}$		96		
	$10^{-3}$	360	116		
16. 2-Benzoxo-2-methyl-1-dimethylamino-butane	0	360	103		
	$10^{-5}$		113		
	$10^{-4}$		114		
	$10^{-3}$	348	111		
17. Cocaine	0	360	107		
	$10^{-5}$		91		
	$10^{-4}$		95		
	$10^{-3}$	371	113		

counteract the bacteriostatic action of sulfapyridine on *B. coli* grown in the simple medium of Kalmanson and Bronfenbrenner.<sup>2</sup>

Three local anaesthetics derived from p-aminobenzoic acid and 3 local anaesthetics not derived from p-aminobenzoic acid were also tested for their ability to counteract the bacteriostatic action of sulfathiazole on *B. coli* in the Kalmanson and Bronfenbrenner medium and of sulfathiazole on *Staphylococcus aureus* grown in an artificial medium. The Lewis strain of *B. coli*, and Strain 679 of *Staphylococcus aureus*, originally obtained from Dr. H. M. Powell, were employed as test organisms.

For the *B. coli* experiments, each culture tube received 2 cc of Kalmanson and Bronfenbrenner's solution A and 2 cc of solution

<sup>2</sup> Kalmanson, G., and Bronfenbrenner, J., *J. Gen. Physiol.*, 1939, **23**, 203.

TABLE II.

Effect of 3 Local Anaesthetics Derived from p-Aminobenzoic Acid (Nos. 2-4) and 3 Local Anaesthetics Not Derived from p-Aminobenzoic Acid (Nos. 5-7) on Bacteriostasis of *B. coli* and *Staphylococcus aureus* by Sulfathiazole. The Extent of Growth (24 hr for *B. coli* and 48 hr for *S. aureus*) is expressed as in Table I.

Local Anaesthetic	Conc. local anesthetic	Growth of <i>B. coli</i> in various molar conc. of sulfathiazole				Growth of <i>S. aureus</i> in various molar conc. of sulfathiazole			
		0	10 <sup>-4</sup>	8 x 10 <sup>-4</sup>	0	10 <sup>-4</sup>	4 x 10 <sup>-4</sup>	1.6 x 10 <sup>-3</sup>	
None — controls	moles per l								
1. p-Aminobenzoic Acid	0	372	114	108	413	70	65	62	
	10 <sup>-6</sup>		284	126		358	87	66	
	10 <sup>-5</sup>		387	274		401	390	112	
	10 <sup>-4</sup>	365	370	414	401		393	309	
2. $\beta$ -Diethylaminoethyl-p-aminobenzoate	10 <sup>-5</sup>		109	108		395	94	48	
	10 <sup>-4</sup>		298	120		390	424	218	
	10 <sup>-3</sup>	358	364	291	392	404	402	420	
3. $\gamma$ -Dimethylamino- $\alpha,\beta$ -dimethylpropyl-p-aminobenzoate	10 <sup>-5</sup>		120	100		86	90	78	
	10 <sup>-4</sup>		263	119		410	108	64	
	10 <sup>-3</sup>	340	360	234	400	397	420	168	
4. $\gamma$ -Diethylamino- $\beta,\beta$ -dimethylpropyl-p-aminobenzoate	10 <sup>-5</sup>		140	107		106	79	67	
	10 <sup>-4</sup>		122	126		429	121	72	
	10 <sup>-3</sup>	379	284	129	412	411	386	195	
5. $\gamma$ -(2-methyl-piperidino)-propylbenzoate	10 <sup>-5</sup>		110	94		82	73	66	
	10 <sup>-4</sup>		97	98		77	56	60	
	10 <sup>-3</sup>	360	80	100	391	90	71	67	
6. $\gamma$ -Diethylaminopropyl cinnamate	10 <sup>-5</sup>		121	102		85	89	70	
	10 <sup>-4</sup>		113	110		78	81	47	
	10 <sup>-3</sup>	327	96	102	316	67			
7. 2-Butyloxyquinoline carboxylic acid-4-diethylethylenediamide	10 <sup>-5</sup>		116	105					
	10 <sup>-4</sup>		114	107					
	10 <sup>-3</sup>	350	13	22					

B, followed in order by 2 cc of sulfapyridine solution in sterile distilled water and 1 cc of local anaesthetic solution in sterile distilled water to give the concentrations shown in the tables, 0.4 cc of sterile glucose solution to give a final concentration of 2.5 mg per cc, and sterile distilled water to make 7.9 cc. An inoculum of 0.1 cc ( $5 \times 10^8$  organisms) of 24 hr culture of *B. coli* in the simple medium was added, the inoculated tubes then being incubated at 38°C. Under these conditions, the original inoculum of  $6 \times 10^6$  organisms per cc increased to a maximum of about  $6 \times 10^9$  organisms per cc in 8 to 10 hours, remaining virtually constant thereafter. The extent of growth was estimated, after 24-hour incubation, by photoelectric density readings on an Exton Scopometer, the instrument having been calibrated by making density readings on suspensions of known population. A density reading of 100 corresponds to a count of approximately  $1 \times 10^9$  organisms per cc, a reading of 300 to approximately  $6 \times 10^9$  organisms per cc. Though complete suppression of growth by sulfapyridine can be obtained by use of smaller inocula, the present conditions were deliberately selected to enable sulfapyridine and sulfathiazole to exhibit a pronounced bacteriostatic effect at a concentration of the same order as that which usually prevails in the blood of animals given therapeutic doses of the drug.

For the *Staphylococcus aureus* experiments, the artificial medium contained the following materials per cc: 18 amino acids, in approximately the ratio of hydrolysed casein,<sup>3</sup> to give a total final concentration of 2 mg; glutathione, 100 µg; pantothenic acid, 10 µg; nicotinamide, 50 µg; thiamine, 10 µg;  $\text{Na}_2\text{HPO}_4$ , 1.6 mg;  $\text{MgSO}_4$ ,  $\text{K}_2\text{SO}_4$ ,  $\text{CaCl}_2$ , each 0.16 mg;  $\text{NaCl}$ , 1.6 mg;  $\text{NaHCO}_3$ , 0.8 mg; glucose, 10 mg. The *Staphylococcus aureus*, which had been cultivated on agar slants, was inoculated into beef extract broth and allowed to grow 16 hr at 38°C. This bacterial suspension was then diluted 1:10 with the artificial medium and 0.1 cc of the diluted suspension was transferred to 7.9 cc of the artificial medium under test. This inoculum of approximately  $5 \times 10^5$  organisms per cc increased to a maximum of approximately  $12 \times 10^9$  organisms per cc in the test period of 48 hr.

All the local anaesthetics were commercial samples procured on the open market; they were all used in the form of their hydrochlorides. Where necessary, the conversion of the commercial samples to the pure hydrochlorides was made in this laboratory; the authors are indebted to Dr. E. C. Kleiderer for his collaboration in such purification.

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<sup>3</sup> Wooley, D. W., and Hutchings, B. L., *J. Bact.*, 1940, **39**, 287.



Typical results with each of 16 local anaesthetics alone and in conjunction with sulfapyridine are shown in Table I. It will be noted that each of the 7 p-aminobenzoic acid derivatives partially or completely blocked the bacteriostatic effect of the sulfapyridine; the blocking effect was reduced but not eliminated by substitution in the amino group or esterification of the carboxyl group of p-aminobenzoic acid. None of the 9 local anaesthetics not derived from p-aminobenzoic acid displayed any antagonism to the sulfapyridine. In these experiments, p-aminobenzoic acid could counteract the effect of approximately 200 to 500 molecular equivalents of sulfapyridine; from Table I the corresponding ratios for local anaesthetics nos. 2, 3, and 4 were, respectively, approximately 20, 40, and 2.

Each of the 3 p-aminobenzoic acid derivatives blocked the action of sulfathiazole; none of the non-p-aminobenzoic acid derivatives displayed any antagonism to sulfathiazole (Table II).

*Summary.* Each of 7 local anaesthetics derived from p-aminobenzoic acid partially or completely blocked the *in vitro* bacteriostatic effect of sulfapyridine upon a strain of *B. coli* grown in a simple medium. None of 9 local anaesthetics not derived from p-aminobenzoic acid displayed any antagonism to the sulfapyridine. Each of 3 local anaesthetics derived from p-aminobenzoic acid blocked the bacteriostatic effect of sulfathiazole on *B. coli* and *Staphylococcus aureus*. None of 3 local anaesthetics not derived from p-aminobenzoic acid displayed any antagonism to the sulfathiazole.

### 13199

#### The Assay *Recovery* of Prothrombin Added to Plasma.

JOHN H. FERGUSON.

*From the Department of Pharmacology, University of Michigan, Ann Arbor.*

In the promulgation of current methods of prothrombin assay<sup>1, 2</sup> there has been some investigation of plasma dilutions but no direct experiments to show the ability to *recover* prothrombin added to plasma. This is important in connection with the possibility that

<sup>1</sup> Quick, A. J., *J. Am. Med. Assn.*, 1938, **110**, 1658.

<sup>2</sup> Brinkhous, K. M., *Medicine*, 1940, **19**, 329.

clot-inhibitors<sup>3-6</sup> may complicate the interpretation of the assay. It is recognized, for instance, that heparin interferes with prothrombin determinations<sup>2</sup> and heparin can act both as an antiprothrombin<sup>6</sup> and as an immediate antithrombin,<sup>5</sup> in the presence of the respective plasma co-factors. The natural antithrombins also include a progressive thrombinolytic factor.<sup>4</sup> As an example of the kind of interference which may be expected, it has recently been found that a series of prothrombin dilutions, subsequently activated with  $\text{Ca}^{++}$  and brain thromboplastin, gives more than the theoretical thrombin yield, as determined from the clotting-times for purified fibrinogen, using a similar dilution series of the same (full-strength) thrombin as the standard of reference.<sup>6</sup> In the absence of equivalence it is, of course, impossible to assay prothrombin *both* in terms of an arbitrarily fixed clotting-time *and* a definite thrombin dilution value. Inherent in the clinical tests is a false assumption that inhibitors do not influence the results obtained. Actually, there must be a rôle for the natural inhibitors of the types mentioned. The Iowa workers<sup>2</sup> recognized only the progressive antithrombins and succeeded in controlling these by dilution. It is an unproved assumption that immediate antithrombins and antiprothrombins do not interfere with the prothrombin assay. In the following experiment, in which a prepared prothrombin is assayed in the presence of plasma, these problems are readily brought to test.

*Assay of Prothrombin and Plasma. Method.* A Howell-type prothrombin solution<sup>7</sup> is converted into thrombin by the use of 1/20 vol. N/10  $\text{CaCl}_2$  and a similar amount of saline emulsion of frozen dog brain (thromboplastin). The thrombic mixture is held at 10°C and 0.5 cc samples tested (*A*) at the cited intervals, by timing the clotting after adding to 1.0 cc fibrinogen (temp. = 37°C, pH = 7.5). Similar tests are made on citrated dog plasma which has been heated to 56°C, to defibrinate, and diluted with 0.9% NaCl. Data are given for several dilutions of prothrombin and plasma and mixtures of both.

Either the full-strength thrombin or that from the diluted prothrombin (activated) can be used as the standard of reference. Serial dilutions of the thrombin are made and the clotting-times for fibrinogen, under the above uniform conditions, are used for

<sup>3</sup> Ferguson, J. H., *Am. J. Physiol.*, 1940, **130**, 759.

<sup>4</sup> Glazko, A. J., and Ferguson, J. H., *J. Gen. Physiol.*, 1940, **24**, 169.

<sup>5</sup> Glazko, A. J., and Ferguson, J. H., *Am. J. Physiol.*, 1941 (in press).

<sup>6</sup> Ferguson, J. H., and Glazko, A. J., *Am. J. Physiol.*, 1941 (in press).

<sup>7</sup> Ferguson, J. H., *J. Lab. Clin. Med.*, 1938, **24**, 273.

the reference curve (*B*). The *optimal* clotting-times for the prothrombin and plasma activation series are referred to the thrombin dilution curve, preferably plotted as reciprocal C. T. [ $r = 1000 \div \text{C. T. (seconds)}$ ] against percentage concentration (in terms of original strength). This gives the "recovery" values cited in *C*.

*Data* (Table I). *Exp. 2*. The increase (to 16%) over the theoretical (10%) is due to dilution of natural antiprothrombin, according to the interpretation previously submitted.<sup>6</sup> *Exp. 3, 4, 6*. In these cases, the 5-minute test is already past the optimum potency because of thrombic destruction by progressive (serum-) antithrombin.<sup>4</sup> The true equivalence, therefore, is greater than the figures obtained from the clotting-times.

*Exp. 6*. The apparent inactivation rate is slower than in *Exp. 4*, presumably due to the greater thrombin yield because of the added prothrombin. Even allowing for the uncertainty due to the presence of progressive antithrombin, there is an excess over the theoretical which points to a natural antiprothrombin in the plasma. *Exp. 7, 8*. Here the plasma dilution (50-100 x) is sufficient to ensure a stable thrombin (*i. e.*, practically no progressive antithrombin). When the antiprothrombic factor is allowed for, by using the referred (corrected) thrombin equivalent, the "recovery" values check perfectly. They are practically as good, *viz.* 116 (117) and 85 (84.5) if the

TABLE I.  
"Recovery" of Prothrombin Added to Diluted Plasmas.  
A. Activation Curves (10°C). Clotting-times (sec.) at 37°C. pH = 7.5.

T	Source of thrombin	5'	15'	30'	60'
1.	Prothrombin (1:1)	9"	9"	9"	9"
2.	" (1:10)	120"	35"	30"	33"
3.	*Plasma (1:1)	9"	25"	125"	1800"
4.	* " (1:10)	12"	15"	22"	35"
5.	* " (1:100)	235"	40"	40"	40"
6.	* " (1:10) + Pro. (1:10)	12"	15"	18"	28"
7.	* " (1:50) + Pro. (1:5)	80"	34"	34"	34"
8.	* " (1:100) + Pro. (1:10)	22"	21"	21"	21"

\* Plasma defibrinated by heating (56°C). Equal volumes of plasma and prothrombin (Pro.) were mixed in 6, 7, 8.

B. Thrombin ( $T_1$ ) Dilution Curve = standard of reference.

Dilution	1:1	1:2	1:4	1:8	1:16	1:32	1:64
Clotting-time	9"	14"	22"	35"	52"	80"	113"

C. Prothrombin "Recovery" (Thrombin Percentage):  
Theoretical values in parentheses.

	1.	2.	3.	4.	5.	6.	7.	8.
%	100	16(10)	>100	>65	10	>65(>40)	26(26)	13(13)

1:10 (pro) thrombin is used as the starting point for the standard of reference. On another occasion, the prothrombin "recovery" (theoretical in parenthesis) in 2 similar experiments gave (a) 80 (85), (b) 70.5 (70).

*Comment.* In spite of the demonstrated possibilities of interference by clot inhibitors, the ability to obtain such excellent prothrombin *recovery* shows that these inhibitors are under control, notwithstanding the empirical nature of the assay method. The data confirm the Iowa workers' observation<sup>2</sup> that dilution removes the effects of progressive antithrombins. Immediate antithrombins<sup>5</sup> and antiprothrombins<sup>6</sup> do not interfere, although they are undoubtedly present. The latter, indeed, are demonstrated by analysis of the data. Consideration of the reference standard suggests an explanation. The comparisons are made on the basis of *clotting-times*. Thus the arbitrary thrombin "percentage" assumes a new significance in that it is automatically corrected for differences in the equivalence of thrombin and prothrombin. The only remarkable point is that the same correction seems to hold for diluted plasma and the prothrombin, which was prepared some weeks previously from another animal. The values obtained, of course, are not strictly measures of prothrombin in terms of thrombin but rather in terms which tacitly include variables imposed by the inhibitors present in both. It is justifiable to conclude that the natural inhibitors do not vary under the circumstances investigated. However, this might not hold under other conditions and it is conceivable that certain clinical anomalies of "prothrombin" clotting-times may reside, not in the prothrombin *per se*, but in the associated inhibitor variables. These conditions obtain in heparinized plasma and it is highly improbable, on the basis of recently published studies<sup>5, 6</sup> that they can be overcome by simple dilution or the excess of thromboplastin, upon which the modern prothrombin methods essentially depend.





## SECRETARY'S REPORT

April 1, 1940-March 31, 1941

The annual meetings of the standing committees of the Society were held April 18, 1941. These included Membership Committee, Board of Editors, Council, and Section Secretaries.

*Finances.* There was no deficit for the fiscal year 1940-41. For the next year we expect both increase in costs of publication and decreased income from foreign subscribers, and the inability of foreign members to pay their annual dues. To meet this situation the Council voted to *increase the dues of members from \$4.00 to \$4.50*. During the past year members have been charged for excess space 50% of cost. It is hoped that the increased dues may make it possible to charge members at less than 50% of cost.

*Membership.* Applications for membership are transmitted by mail to the nine members of the National Membership Committee. Each applicant is nominated by one of the nine largest sections of the Society. If the Committee is not unanimous, but more than two-thirds of the Committee approve, the application is reconsidered by the Committee both by mail and at the annual meeting. Such applications are then considered by the Council. If approved all applicants are immediately notified. For this procedure to be consummated it is necessary for *applications to be in the Secretary's office by December 15*. One hundred and eleven applications were approved April 18, 1941.

Doctors C. M. Jackson, H. S. Jennings, and R. Hunt were elected members emeritus.

The following resignations were accepted with regret: Doctors L. Baker, C. Binger, G. E. Coghill, H. Downes, C. Hartman, H. S. Reichle, and R. Tunnicliff.

Six members (in the U. S.) were declared in arrears and dropped from the membership list.

*Foreign members* find considerable difficulty either on account of exchange rates or inability to send money out of their country. The Council voted that (1) *members outside the U. S. shall not be dropped for arrears this year*. (2) *The PROCEEDINGS will be sent to members outside the U. S. with or without payment of dues, for the coming fiscal year*. It is hoped that our finances at the end of the year may make it possible to help our colleagues abroad further.

*South American Scientists.* The Council voted that (1) our present procedures provide adequate methods for election to membership of properly qualified scientists in South American countries. (2) Since there is no adjoining Sectional Membership Committee, such applications shall be referred for endorsement to a committee of members selected by the President and Secretary. This Committee shall have knowledge of conditions and of

workers in South America. (3) The Society is not ready to initiate steps to form a Section outside the United States.

*Nomination of National Officers.* In accordance with the by-laws The Council nominated the national officers to serve for the next two years. These nominations were based on the principle proposed by the Editorial Board that each editor should serve for not more than five years in any one term, thus providing a healthy rotation of office. The Council wished to express its and the Society's appreciation of the splendid work of such retiring editors by nominating them as national officers. The Council nominated the following:

President, W. deB. MacNider, W. J. Meek; Vice-President, C. H. Danforth, S. B. Hooker, A. M. Pappenheimer; Secretary-Treasurer, A. J. Goldforb; Council Members at large, D. W. Bronk, E. M. K. Geiling, A. B. Hastings, J. M. Hayman, Jr.

The President appointed Doctors L. G. Barth, W. H. Chambers, W. Etkin, M. Gregersen and L. T. Webster as Board of Tellers. They found a very close vote. They report the following elected:

<i>President</i>	W. deB. MacNider
<i>Vice-President</i>	C. H. Danforth
<i>Secretary-Treasurer</i>	A. J. Goldforb
<i>Council</i>	E. M. K. Geiling
	A. B. Hastings

*Rocky Mountain Section.* Application to form a new Section near Denver, Colo., to be known as the Rocky Mountain Section was approved.

*Advisory Committee.* Since the officers of the Society are so centralized and spread over large distances, and since under war conditions a group judgment and prompt action have been and may well be necessary, the Council voted to appoint a committee to consult with the Secretary-Treasurer in emergencies. The following were appointed: Doctors H. S. Gasser, H. W. Smith, P. E. Smith.

*Changes in the By-Laws.* These changes, relatively minor, but desirable in view of our experience, were approved by a committee on recommendation of the Council, and submitted to the members by mail and approved.

*Change in Format of PROCEEDINGS.* It was proposed, chiefly for reduced cost, to publish the PROCEEDINGS in two columns per page. The matter was referred to a committee for further study.

The officers on behalf of the Society wish to express their appreciation of the splendid coöperation of:

Dr. Emil Baumann (member) for the care and accuracy, and high merit of the indices of the PROCEEDINGS for the past academic year.

Mr. H. G. Friedman (non-member) for continuous and expert advice on the investments of the Society, during a particularly difficult year.

Mr. William Girden (non-member) for expert advice on non-legal phases of our mortgages.

Mr. Leon Leighton (non-member) for advising on legal phases in connection with mortgages owned by the Society.

PAST OFFICERS

<i>Date</i>	<i>President</i>	<i>Vice-President</i>	<i>Secretary</i>	<i>Treasurer</i>
1903-04	S. J. Meltzer	W. H. Park	W. J. Gies	G. N. Calkins
1904-05	S. J. Meltzer	J. Ewing	" "	" "
1905-06	E. B. Wilson	E. K. Dunham	" "	" "
1906-07	S. Flexner	E. K. Dunham	" "	" "
1907-08	S. Flexner	T. H. Morgan	" "	" "
1908-09	F. S. Lee	T. H. Morgan	" "	G. Lusk
1909-10	F. S. Lee	W. J. Gies	E. L. Opie	" "
1910-11	T. H. Morgan	W. J. Gies	" "	" "
1911-12	T. H. Morgan	P. A. Levene	G. B. Wallace	" "
1912-13	J. Ewing	P. A. Levene	" "	C. Norris
1913-14	J. Ewing	C. W. Field	H. C. Jackson	" "
1914-15	G. Lusk	W. J. Gies	" "	J. R. Murlin
1915-16	G. Lusk	G. N. Calkins	H. C. Jackson	" "
1916-17	J. Loeb	W. J. Gies	" "	" "
1917-19	W. J. Gies	J. Auer	" "	" "
1919-21	G. N. Calkins	G. B. Wallace	" "	" "
1921-23	G. B. Wallace	J. W. Jobling	" "	" "
1923-24	H. C. Jackson	J. W. Jobling	V. C. Myers	" "
1924-25	H. C. Jackson	J. W. Jobling	A. J. Goldforb	" "
1925-27	J. W. Jobling	S. R. Benedict	" "	" "
1927-29	S. R. Benedict	P. Rous	" "	" "
1929-30	P. Rous	D. Marine	" "	" "
1930-31	P. Rous	D. J. Edwards	" "	" "
1931-32	D. J. Edwards	A. R. Dochez	" "	" "
1932-34	A. R. Dochez	E. L. Opie	" "	" "
1934-36	E. L. Opie	P. E. Smith	" "	" "
1936-37	P. E. Smith	E. F. DuBois	" "	" "
1937-39	H. S. Gasser	J. T. Wearn	" "	" "
1939-40	J. T. Wearn	C. D. Leake	" "	" "
1940-41	J. T. Wearn	C. D. Leake	" "	" "

SECTIONAL MEETINGS AND MEMBERSHIP

*Cleveland, Ohio*

Chairman: E. Megrail. Secretary: W. E. Hambourger. Members: 43.

Meetings: Western Reserve University, November 8, 1940  
 December 13, 1940  
 January 10, 1941  
 February 14, 1941  
 March 14, 1941  
 April 11, 1941

*District of Columbia*

Chairman: D. B. Jones. Secretary: H. M. Dyer. Members: 46.

Meetings: U. S. Public Health Service, December 5, 1940  
 February 6, 1941  
 George Washington University, April 3, 1941  
 Cosmos Club, June 12, 1941

*Illinois*

Chairman: K. K. Jones. Secretary: A. Weil. Members: 136.  
 Meetings: University of Illinois Medical School, October 22, 1940  
 University of Chicago, December 10, 1940  
 Northwestern University, January 21, 1941  
 University of Chicago, March 4, 1941  
 May 20, 1941

*Iowa*

Chairman: E. G. Gross. Secretary: T. L. Jahn. Members: 37.  
Meetings: State University of Iowa, February 27, 1941  
May 9, 1941

*Minnesota*

Chairman: F. H. Scott. Secretary: F. H. Scott. Members: 49.  
Meetings: University of Minnesota, November 20, 1940  
January 15, 1941  
March 19, 1941  
May 21, 1941

*Missouri*

Chairman: A. S. Gilson, Jr. Secretary: W. H. Griffith. Members: 54.  
Meetings: Washington University Medical School, October 9, 1940  
St. Louis University Medical School, November 13, 1940  
Washington University Medical School, February 12, 1941  
St. Louis University Medical School, March 12, 1941  
Washington University Medical School, May 14, 1941

*New York*

Chairman: J. C. Hinsey. Secretary: P. Reznikoff. Members: 453.  
Meetings: Rockefeller Institute, November 13, 1940  
Memorial Hospital, February 26, 1941  
New York Academy of Medicine, May 14, 1941

*Pacific Coast*

Chairman: C. A. Kofoid. Secretary: C. Weiss. Members: 100.  
Meetings: Stanford Lane Hospital, December 4, 1940  
University of California, February 5, 1941  
Mount Zion Hospital, March 6, 1941  
Stanford University, May 3, 1941

*Peiping, China*

Chairman: A. B. Fortuyn. Secretary: F. T. Chu. Members: 32.  
Meetings: Peiping Union Medical College, February 12, 1941

*Southern*

Chairman: A. E. Casey. Secretary: W. A. Sodeman. Members: 41.  
Meetings: Tulane University, November 1, 1940  
Louisiana State University, December 20, 1940  
Tulane University, February 7, 1941  
Louisiana State University, May 23, 1941

*Southern California*

Chairman: E. Bogen. Secretary: M. S. Dunn. Members: 42.  
Meetings: University of Southern California, October 10, 1940  
Occidental College, Los Angeles, December 11, 1940  
Pasadena, Calif., June 20, 1941

*Western New York*

Chairman: E. F. Adolph. Secretary: H. C. Hodge. Members: 59.

Meetings: N. Y. State Agri. Exp. Station, October 19, 1940

University of Rochester, December 14, 1940

Clifton Springs Sanitarium, February 15, 1941

*Wisconsin*

Chairman: R. M. Meyer. Secretary: H. P. Rusch. Members: 40.

Meetings: Marquette University, April 4, 1941

## MEMBERSHIP

Members, March 31, 1940.....	1465	
Elected during year.....	100	
Total .....		1565
Resignations .....	3	
Deaths .....	13	16
Total Membership, March 31, 1941.....		1549
Membership: 1931      1941		
1062      1549		
Subscriptions, March 31, 1941.....		614

## DEATHS OF MEMBERS

The Council records with regret the deaths of the following members:  
 Doctors F. G. Banting, E. C. Dickson, J. G. Dusser de Barenne, C. P. Fitch,  
 J. G. Fitzgerald, G. K. Noble, R. Pearl, D. G. Richey, M. S. Rose, and  
 H. Zinsser.



## TREASURER'S REPORT

April 1, 1940-April 1, 1941

Balance on hand, April 1, 1940..... \$ 4,756.42

### *Income*

Dues .....	\$ 6,347.10
Reprints .....	3,975.96
Space .....	2,804.90
Cuts .....	743.97
Changes .....	49.63
Subscriptions .....	5,066.86
Back No. ....	705.50
Interest from special account and misc.....	21.79
	\$19,715.71

Transfer of interest from Surplus and Endowment Funds .....	800.24
Endowment Fund Temporarily in Corn Exchange Bank .....	515.20

Total Cash Available, April 1, 1940-April 1, 1941.....	\$25,787.57
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### *Disbursements*

Printing .....	\$11,033.54
Reprints .....	4,085.25
Cuts .....	1,030.28
	\$16,149.07
Office Supplies, Postage and Telephone.....	830.41
Salary .....	2,205.00
Storage and Insurance .....	74.75
Misc. ....	207.35
	\$ 3,317.51

	\$19,466.58
Cash Balance, April 1, 1941.....	\$ 6,320.99
	\$25,787.57

### SUMMARY

Income (net) .....	\$19,715.71
Disbursements (net) .....	19,466.58
	Surplus \$ 249.13

Accounts receivable—\$1,566.94

Accounts payable       None

## FUNDS

*Endowment Fund*

April 1, 1940 .....	\$17,965.95	
Interest to April 1, 1941.....	747.57	
	<hr/>	\$18,713.52
Interest transferred to Corn Exchange Bank.....		300.00
		<hr/>
		\$18,413.52
Invested in New York Title and Mortgage Co.....	\$ 5,760.00	
Title Guarantee and Trust Co.....	2,000.00	
Lawyers Mortgage Co.....	1,500.00	
Bowery Savings Bank.....	2,991.83	
U. S. Savings Bonds.....	1,925.00	
Industrial Bonds .....	3,721.49*	
Temporarily in Corn Exchange Bank.....	515.20	
	<hr/>	\$18,413.52

*Surplus Fund*

April 1, 1940.....	\$11,424.40	
Interest to April 1, 1941.....	464.93	
	<hr/>	\$11,889.33
Interest transferred to Corn Exchange Bank.....		500.24
		<hr/>
		\$11,389.09
Invested in Title Guarantee and Trust Co.....	2,820.60	
Harlem Savings Bank.....	769.30	
U. S. Savings Bonds.....	1,925.00	
Industrial Bonds .....	5,874.19*	
	<hr/>	\$11,389.09

*Life Membership Fund*

Invested in R. R. Federal Savings and Loan.....	\$	75.00
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\* Cost values.

**Auditors' Report**

June 6, 1941

We the undersigned have this day examined the Treasurer's report and find it to agree with the books of the Society. We believe that the records of the financial transactions are accurate and in good order.

(Signed) ISIDOR GREENWALD  
MCKEEN CATTELL

# MEMBERS' LIST

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## HONORARY MEMBERS

Flexner, Simon.....	Rockefeller Inst.
Howell, William H.....	Johns Hopkins Univ.
Porter, William.....	Harvard Univ.
Richet, Charles.....	Paris, France
Von Muller, Friedrich.....	Munich, Germany

## MEMBERS

<b>A</b> bramson, D. I.....	May Inst. Med. Research, Cincinnati
Abramson, H. A.....	Coll. Physicians and Surgeons, New York
Abt, Arthur F.....	Northwestern Univ.
Adams, A. Elizabeth.....	Mount Holyoke Coll.
Adams, William E.....	Univ. of Chicago
Addis, Thomas.....	Stanford Univ. Med.
Adlersberg, D.....	Beth Israel Hosp., N. Y.
Adolph, E. F.....	Univ. of Rochester Med.
Adolph, W. H.....	Peiping, China
Alexander, Harry L.....	Washington Univ.
Allen, Bennet M.....	Univ. of Calif., L. A.
Allen, Edgar.....	Yale Univ.
Allen, William F.....	Univ. of Oregon
Alles, G. A.....	Pasadena, Calif.
Almquist, H. J.....	Univ. of Calif.
Alt, Howard L.....	Northwestern Univ. Med.
Althausen, T. L.....	Univ. of Calif. Med.
Altshule, M. D.....	Beth Israel Hosp., Boston
Altshuler, S. S.....	Wayne Univ. Med.
Alvarez, Walter C.....	Mayo Clinic
Alving, A. S.....	Univ. of Chicago
Amberg, Samuel.....	Mayo Clinic
Amberson, W. R.....	Univ. of Maryland Med.
Amoss, Harold L.....	Rockefeller Inst.
Anderson, Dorothy H.....	Coll. of Phys. and Surg., N. Y.
Anderson, H. H.....	Peiping Union Med. Coll.
Anderson, John E.....	Univ. of Minn.
Anderson, John F.....	E. R. Squibb & Son
Anderson, Rudolph J.....	Yale Univ.
Anderson, William E.....	Rockville, Conn.
Andrews, Edmund.....	Los Angeles, Calif.
Andrus, E. C.....	Johns Hopkins Univ.
Andrus, W. deW.....	Cornell Univ. Med. Coll.
Ansbacher, Stefan.....	New York City
Antopol, William.....	Beth Israel Hosp., Newark, N. J.
Apperly, Frank L.....	Med. Coll. of Va.

Armstrong, Charles.....	National Inst. of Health, Washington
Arnold, Lloyd.....	Univ. of Ill.
Aronson, J. D.....	Henry Phipps Inst.
Asdell, S. A.....	Cornell Univ.
Asher, Leon.....	Berne, Switzerland
Ashman, Richard.....	Louisiana State Univ.
Asmundson, V. S.....	Univ. of Calif.
Atchley, D. W.....	Presbyterian Hosp., N. Y. C.
Aub, Joseph C.....	Huntington Memorial Hosp., Boston
Auer, John.....	St. Louis Univ.
Austin, J. Harold.....	Univ. of Pa.
Avery, B. F.....	Am. Univ. of Beirut
Avery, O. T.....	Rockefeller Inst., N. Y. C.
Avery, Roy C.....	Vanderbilt Univ.
Aycock, W. L.....	Harvard Med.

<b>B</b> abkin, Boris P.....	McGill Univ.
Bachem, Albert.....	Univ. of Ill. Med. Coll.
Baehr, George.....	Mt. Sinai Hosp., N. Y. C.
Bagg, Halsey J.....	Memorial Hosp., N. Y. C.
Bahrs, Alice M.....	Portland, Ore.
Bailey, Cameron V.....	N. Y. Post-Graduate Med.
Bailey P. V.....	Univ. of Illinois Med.
Baitsell, George A.....	Yale Univ.
Bakwin, Harry.....	N. Y. Univ. Med. Coll.
Baldwin, Francis M.....	Univ. of S. Calif.
Ball, G. H.....	Univ. of Calif., L. A.
Ball, H. A.....	San Diego, Calif.
Balls, A. K.....	U. S. Dept. of Agr.
Barach, Alvan L.....	Coll. of Phys. and Surgeons, N. Y.
Barber, W. Howard.....	New York Univ. Med.
Barbour, Henry G.....	Yale Univ.
Barer, Adelaide P.....	State Univ. of Iowa
Barker, S. B.....	Cornell Med. Coll.
Barlow, O. W.....	Rensselaer, N. Y.
Barnett, George D.....	Stanford Univ.
Barr, David P.....	Washington Univ.
Barron, E. S. G.....	Univ. of Chicago
Barth, L. G.....	Columbia Univ.
Bartley, S. H.....	Washington Univ.
Bass, Charles.....	Tulane Univ.
Bast, T. H.....	Univ. of Wisconsin
Bates, R. W.....	Carnegie Inst. of Washington
Batterman, R. C.....	New York Univ. Med.
Bauer, J. H.....	Rockefeller Inst.
Bauman, Louis.....	Presbyterian Hosp., N. Y. C.
Baumann, E. J.....	Montefiore Hosp., N. Y. C.
Baumberger, J. Percy.....	Stanford Univ.
Bayne-Jones, S.....	Yale Univ.
Bazett, H. C.....	Univ. of Pa.

Bean, John W.....	Univ. of Mich.
Beard, H. H.....	Louisiana State Univ.
Beard, J. W.....	Duke Univ.
Beard, P. J.....	Stanford Univ.
Beck, Claude S.....	Western Reserve Univ.
Becker, E. R.....	Iowa State Coll.
Beckman, Harry.....	Marquette Univ. Med.
Beckwith, T. D.....	Univ. of Calif., L. A.
Behre, Jeannette A.....	Wilmette, Ill.
Belding, David L.....	Boston Univ.
Bender, M. B.....	Mt. Sinai Hosp., N. Y.
Bengston, Ida A.....	National Inst. of Health, Washington
Berg, B. N.....	Columbia Univ.
Berg, C. P.....	State Univ. of Iowa
Berg, William N.....	N. Y. City
Bergeim, Olaf.....	Univ. of Ill.
Bergmann, Max.....	Rockefeller Inst.
Bernhard, Adolph.....	Lenox Hill Hosp., N. Y. City
Bernthal, T. G.....	Vanderbilt Univ.
Berry, George P.....	Univ. of Rochester Med.
Beutner, R.....	Hahnemann Med. Coll., Philadelphia
Bierman, W.....	Mt. Sinai Hosp., N. Y.
Bieter, Raymond N.....	Univ. of Minn.
Bills, C. E.....	Mead, Johnson and Co., Evansville, Ind.
Bing, Franklin C.....	Am. Med. Assn., Chicago
Birkhaug, Konrad E.....	Geofysisk Inst., Bergen, Norway
Bishop, George H.....	Webster Groves, Mo.
Bishop, Katharine S.....	Univ. of Calif.
Blackfan, K. D.....	Harvard Med.
Blair, John E.....	Hosp. for Joint Diseases, N. Y.
Blake, F. G.....	Yale Univ.
Blalock, Alfred.....	Vanderbilt Univ. Med.
Blatherwick, Norman R.....	Metropolitan Life Insurance Co., N. Y. City
Blau, Nathan F.....	Cornell Univ. Med. Coll.
Blinks, L. R.....	Stanford Univ.
Bliss, Sidney.....	Tulane Univ.
Bloch, Robert G.....	Univ. of Chicago
Block, Richard J.....	N. Y. State Psychiatric Inst.
Bloom, William.....	Univ. of Chicago
Bloomfield, A. L.....	Stanford Univ. Med.
Bloor, W. R.....	Univ. of Rochester
Blount, R. F.....	Univ. of Minn.
Blum, Harold F.....	Washington, D. C.
Blumberg, Harold.....	Johns Hopkins Univ.
Blumgart, H. L.....	Beth Israel Hosp., Boston
Bock, Joseph C.....	Marquette Univ.
Bodansky, A.....	Hosp. for Joint Diseases, N. Y.
Bodansky, Meyer.....	John Sealy Hosp., Galveston, Texas
Bodansky, Oscar.....	New York Univ.
Bodine, J. H.....	State Univ. of Iowa



Bogen, Emil.....	Olive View, Calif.
Boissevain, Charles H.....	Colorado Coll.
Bollman, Jesse L.....	Mayo Clinic
Bonner, James.....	Calif. Inst. of Technology
Boor, Alden K.....	Univ. of Chicago
Boothby, Walter M.....	Kawler Hosp., Rochester, Minn.
Boots, Ralph H.....	Presbyterian Hosp., N. Y.
Borsook, Henry.....	Calif. Inst. of Technology
Bowen, B. D.....	Buffalo Gen. Hosp.
Boyd, Eldon M.....	Queens Univ., Canada
Boyd, Theo. E.....	Loyola Univ.
Boyden, E. A.....	Univ. of Minn. Med.
Bozler, Emil.....	Ohio State Univ.
Bradford, William L.....	Univ. of Rochester
Bradley, H. C.....	Univ. of Wisconsin
Brand, Erwin.....	N. Y. State Psychiatric Inst.
Branham, Sara E.....	National Inst. of Health, Washington
Brewer, George.....	Univ. of Pa.
Brewer, Robert K.....	Syracuse Univ.
Briggs, A. P.....	Univ. of Georgia
Bronfenbrenner, J.....	Washington Univ.
Bronk, D. W.....	Cornell Med. College
Brooks, Clyde.....	Louisiana State Univ.
Brooks, Matilda M.....	Univ. of Calif.
Brooks, S. C.....	Univ. of Calif.
Broun, G. O.....	St. Louis Univ. Med.
Brown, J. Howard.....	Johns Hopkins Univ.
Brown, John B.....	Ohio State Univ.
Brown, Rachel.....	N. Y. State Dept. of Health
Brown, Wade H.....	Rockefeller Inst., Princeton
Browne, J. S. L.....	Royal Victoria Hosp., Montreal
Bruger, Maurice.....	N. Y. Post-Graduate Med.
Brunschwig, Alexander.....	Univ. of Chicago
Buchanan, A. R.....	Univ. of Colo.
Buchanan, Robert E.....	Iowa State Coll.
Buchbinder, W. C.....	Michael Reese Hosp., Chicago
Buell, Mary V.....	Johns Hopkins Univ.
Bulger, H. A.....	Washington Univ.
Bullock, J. G. M.....	Harlem Hosp., N. Y.
Bunting, C. H.....	Univ. of Wisconsin
Burch, George E.....	Rockefeller Inst.
Burch, John C.....	Vanderbilt Univ. Med.
Burdon, Kenneth L.....	Louisiana State Univ.
Burky, Earl L.....	Johns Hopkins Hosp.
Burns, E. L.....	Louisiana State Univ.
Burns, Robert K., Jr.....	Carnegie Inst., Baltimore, Md.
Burr, George O.....	Univ. of Minn.
Burrows, M. T.....	Pasadena, Calif.
Burrows, William.....	Univ. of Chicago
Burstein, C. L.....	N. Y. Univ. Med.

Butcher, E. O.....	Hamilton Coll.
Butt, E. M.....	Univ. of So. Calif.
Butts, Joseph S.....	Oregon State Coll.
Byerly, T. C.....	U. S. Animal Exp. Farm, Beltsville, Md.
Byrne, Joseph.....	Fordham Univ.

<b>C</b> ahill, W. M.....	Univ. of Mich.
Calkins, Gary N.....	Columbia Univ.
Cameron, A. T.....	Univ. of Manitoba
Cannan, Robert K.....	N. Y. Univ.
Cannon, Paul R.....	Univ. of Chicago
Cannon, Walter B.....	Harvard Med.
Cantarow, Abraham.....	Jefferson Med. Coll.
Carey, E. J.....	Marquette Univ.
Carlson, A. J.....	Univ. of Chicago
Carmichael, E. B.....	Univ. of Alabama Med.
Carmichael, L.....	Tufts Coll.
Carr, J. L.....	Univ. of Calif. Hosp.
Carruthers, A.....	Birmingham, England
Casey, Albert E.....	Louisiana State Univ.
Cash, James R.....	Univ. of Va.
Casida, L. E.....	Univ. of Wisconsin
Cattell, McKeen.....	Cornell Univ. Med. Coll.
Cecil, R. L.....	Cornell Univ. Med. Coll.
Cerecedo, L. R.....	Fordham Univ.
Chace, Arthur F.....	N. Y. Post-Graduate Med. Coll.
Chaikoff, I. L.....	Univ. of Calif.
Chambers, Robert.....	New York Univ.
Chambers, William H.....	Cornell Univ. Med. Coll.
Chang, Hsi Chun.....	Peiping Union Med. Coll.
Chang, Hsiao-Chien.....	Hunan, China
Chargaff, Erwin.....	Coll. of Phys. and Surg.
Charipper, H. A.....	New York Univ.
Cheer, S. N.....	W. China Union Univ.
Chen, Graham M.....	Chicago, Ill.
Chen, K. K.....	Eli Lilly and Co., Indianapolis
Chen, T. T.....	Peiping Union Med. Coll.
Cheney, R. H.....	Long Island Univ.
Chidester, F. E.....	Toronto, Can.
Child, C. M.....	Stanford Univ.
Chittenden, R. H.....	Yale Univ.
Chouke, K. S.....	Univ. of Pa.
Chow, B. F.....	Squibb Inst., New Brunswick, N. J.
Christensen, K.....	St. Louis Univ.
Christian, Henry A.....	Peter Bent Brigham Hosp.
Christman, Adam A.....	Univ. of Mich.
Chu, F. T.....	Peiping Union Med. Coll.
Chung, H. L.....	Peiping Union Med. Coll.
Clark, A. J.....	Univ. of Edinburgh, Scotland
Clark, Ada R.....	Coll. of Phys. and Surg.

Clark, Guy W.....	Lederle Lab., Pearl River, N. Y.
Clark, P. F.....	Univ. of Wisconsin
Clarke, Hans T.....	Coll. of Phys. and Surg.
Claude, A.....	Rockefeller Inst.
Clausen, H. J.....	Univ. of Colo. Med.
Claussen, S. W.....	Strong Memorial Hosp., Rochester, N. Y.
Clawson, Benjamin J.....	Univ. of Minn.
Clifton, Charles E.....	Stanford Univ.
Climenko, D. R.....	Rensselaer, N. Y.
Clowes, G. H. A.....	Eli Lilly and Co., Indianapolis
Coca, A. F.....	Oradell, N. J.
Code, C. F.....	Mayo Foundation
Coggeshall, L. T.....	Rockefeller Foundation
Cohen, Barnett.....	Johns Hopkins Med.
Cohen, Martin.....	N. Y. Post-Graduate Med.
Cohen, Milton B.....	St. Alexis Hosp., Cleveland
Cohn, A. E.....	Rockefeller Inst., N. Y.
Cohn, Isidore.....	New Orleans, La.
Cole, Arthur G.....	Univ. of Ill. Med.
Cole, E. C.....	Williams Coll.
Cole, Harold H.....	Univ. of Calif., Davis
Cole, L. J.....	Univ. of Wise.
Cole, Rufus I.....	Rockefeller Inst., N. Y. City
Cole, Warren H.....	Univ. of Ill. Med.
Cole, William H.....	Rutgers Univ.
Collens, William S.....	Brooklyn, N. Y.
Collett, Mary E.....	Western Reserve Univ.
Collier, William D.....	St. Elizabeth's Hosp., Youngstown, O.
Collins, D. A.....	Temple Univ.
Collip, J. B.....	McGill Univ.
Compere, E. L.....	Univ. of Chicago
Conklin, E. G.....	Princeton Univ.
Connor, Charles L.....	Univ. of Calif. Med.
Cook, Charles A.....	Burroughs Wellcome and Co.
Cook, Donald H.....	School of Tropical Med., San Juan, P. R.
Cooke, J. V.....	Washington Univ.
Coombs, Helen C.....	Brooklyn College
Cooper, Frank B.....	W. Penn. Hosp., Pittsburgh
Cope, O. M.....	N. Y. Med. Coll.
Copenhaver, W. M.....	Columbia Univ.
Corey, E. L.....	Univ. of Va.
Cori, Carl F.....	Washington Univ.
Corley, Ralph C.....	Purdue Univ.
Corner, George W.....	Carnegie Inst., Baltimore
Corper, H. J.....	National Jewish Hosp., Denver, Colo.
Co Tui.....	N. Y. Univ. Med. Coll.
Cowdry, E. V.....	Washington Univ.
Cowgill, George R.....	Yale Univ.
Cox, Herald R.....	U. S. Public Health Inst., Hamilton, Mont.
Cox, Warren M., Jr.....	Mead Johnson Co.

Cram, Eloise B.....	Nat. Inst. of Health, Washington
Crampton, C. Ward.....	N. Y. Post-Graduate Med.
Crandall, L. A., Jr.....	Univ. of Tenn.
Creaser, C. W.....	Wayne Univ. Med.
Crile, George W.....	Western Reserve Univ.
Crittenden, Phoebe J.....	George Washington Univ.
Crohn, Burrill B.....	Mt. Sinai Hosp., N. Y.
Csonka, F. A.....	U. S. Dept. of Agr., Washington, D. C.
Cummins, Harold.....	Tulane Univ.
Cunningham, Bert.....	Duke Univ.
Curtis, G. M.....	Ohio State Univ.
Curtis, Maynie R.....	Columbia Univ.
Cutler, Elliott C.....	Peter Bent Brigham Hosp., Boston
Cutting, W. C.....	Stanford Univ. Med.
Cutuly, Eugene.....	Wayne Univ. Med.

<b>D</b> ack, Gail M.....	Univ. of Chicago
Dakin, H. D.....	Ossining, N. Y.
Dalldorf, Gilbert.....	Grasslands Hosp., Valhalla, N. Y.
Dalton, A. J.....	McGill Univ.
D'Amour, F. E.....	Univ. of Denver
Danforth, Charles H.....	Stanford Univ.
Danforth, D. N.....	Evanston, Ill.
Daniel, J. Frank.....	Univ. of Calif.
Daniels, Amy L.....	Univ. of Iowa
Danzer, Charles S.....	N. Y. Med. Coll.
Dautrebande, Lucien.....	Univ. of Liege, Belgium
Davenport, C. B.....	Sta. for Exp. Evolution, Cold Spring Harbor, N. Y.
Davenport, H. A.....	Northwestern Univ. Med.
Davis, D. J.....	Wilmette, Ill.
Davis, H. A.....	Louisiana State Univ.
Davis, J. E.....	Univ. of Vermont
Davis, M. E.....	Univ. of Chicago
Dawson, James A.....	Coll. City of N. Y.
Dawson, M. H.....	Columbia Univ.
Day, A. A.....	Northwestern Univ. Med.
Day, Paul L.....	Univ. of Arkansas Med.
DeBodo, Richard.....	New York Univ. Med.
Decherd, George M.....	Univ. of Texas Med.
DeEds, Floyd.....	Stanford Univ. Med.
DeGowin, E. L.....	State Univ. of Iowa
DeGraff, A. C.....	N. Y. Univ. Med.
Dennis, E. W.....	Amer. Univ. of Beirut
DeRenyi, G. S.....	Univ. of Pa.
De Savitsch, Eugene.....	Washington, D. C.
Detwiler, S. R.....	Columbia Univ.
Deuel, Harry J., Jr.....	Univ. of S. Calif. Med.
Dick, George F.....	Univ. of Chicago
Dieckmann, W. J.....	Univ. of Chicago
Dienes, Louis.....	Massachusetts General Hosp., Boston

Dieuaide, Francis R.	Mass. Gen. Hospital, Boston
Doan, Charles A.	Ohio State Univ.
Dochez, A. R.	Presbyterian Hosp., N. Y. City
Dock, William	Stanford Univ.
Dohan, F. C.	Univ. of Pa.
Doisy, Edward A.	St. Louis Univ.
Dolley, W. L., Jr.	Univ. of Buffalo
Dominguez, R.	St. Luke's Hosp., Cleveland
Domm, L. V.	Univ. of Chicago
Donaldson, J. C.	Univ. of Pittsburgh
Dooley, M. S.	Syracuse Univ.
Dorfman, Ralph I.	Western Reserve Univ.
Dotti, L. B.	New York Med. Coll.
Doubilet, Henry	Mt. Sinai Hosp., N. Y.
Doull, J. A.	Western Reserve Univ.
Drabkin, D. L.	Univ. of Pa.
Dragstedt, Carl A.	Northwestern Univ.
Dragstedt, Lester R.	Univ. of Chicago
Draper, William B.	Univ. of Colo.
Dresbach, M.	Philadelphia, Pa.
Drury, D. R.	Univ. of S. Calif. Med.
Dubin, Harry E.	N. Y. City
DuBois, E. F.	Cornell Univ. Med. Coll.
DuBois, F. S.	Hartford, Conn.
Dubos, Rene J.	Rockefeller Inst.
Duggar, B. M.	Univ. of Wisconsin
Dukes, H. H.	Cornell Univ.
Dunn, Leslie C.	Columbia Univ.
Dunn, Max	Univ. of Calif.
Duran-Reynals, F.	Yale Univ.
Dutcher, R. Adams.	Penn. State Coll.
Duval, C. W.	Tulane Univ.
Du Vigneaud, Vincent	Cornell Univ. Med. Coll.
Dye, Joseph A.	Cornell Univ. Med.
Dyer, Helen M.	George Washington Univ. Med.
Dyer, R. Eugene	National Inst. of Health, Washington

<b>E</b> arle, Wilton R.	National Cancer Inst., Bethesda, Md.
Eastman, N. J.	Johns Hopkins Univ.
Eaton, Alonzo G.	Louisiana State Univ.
Eaton, M. D.	Dept. of Public Health, Berkeley, Calif.
Ebersson, Frederick	Gallinger Municipal Hosp., Washington, D. C.
Ecker, E. E.	Western Reserve Univ.
Eckstein, Henry C.	Univ. of Mich.
Eddy, Walter H.	New York City
Edwards, D. J.	Cornell Univ. Med. Coll.
Edwards, John G.	Univ. of Buffalo
Edwards, Philip R.	Kentucky Agri. Exp. Sta.
Eggston, Andrew A.	N. Y. Manhattan Eye, Ear Hosp.
Eichelberger, Lillian	Univ. of Chicago



Ellinger, F. P.	Montefiore Hosp., N. Y.
Ellis, Max M.	Univ. of Mo.
Elsberg, Charles A.	Neurological Inst., N. Y. City
Elser, W. J.	Cornell Univ. Med. Coll.
Elvehjem, C. A.	Univ. of Wisconsin
Emerson, G. A.	West Va. Univ. Med.
Emerson, Gladys A.	Univ. of Calif.
Emery, F. E.	Univ. of Buffalo
Emge, L. A.	Stanford Univ.
Enders, J. F.	Harvard Univ.
Engle, E. T.	Columbia Univ.
Epstein, A. A.	Mt. Sinai Hosp., N. Y.
Erlanger, Joseph	Washington Univ.
Ernstene, Arthur C.	Cleveland Clinic
Essex, Hiram E.	Mayo Clinic
Etkin, William	Coll. City of N. Y.
Evans, Alice C.	National Inst. of Health, Washington
Evans, Gerald T.	Univ. of Minn.
Evans, Herbert M.	Univ. of Calif.
Everett, M. R.	Univ. of Okla. Med.
Ewing, James	Cornell Univ. Med. Coll.
Eyster, J. A. E.	Univ. of Wise.

<b>F</b> aber, Harold K.	Stanford Univ. Med.
Falk, K. George	N. Y. Univ. Med. Coll.
Famulener, L. W.	Englewood, N. J.
Farmer, Chester	Northwestern Univ.
Farr, L. E.	Wilmington, Del.
Faust, Ernest C.	Tulane Univ.
Fearing, Franklin	Univ. of Calif.
Feil, Harold	Western Reserve Univ.
Fellows, E. J.	Temple Univ.
Feng, T. P.	Peiping Union Med. Coll.
Fenn, Wallace O.	Univ. of Rochester Med.
Ferguson, John H.	Univ. of Mich.
Ferguson, J. K. W.	Univ. of Toronto
Ferraro, Armando	N. Y. State Psychiatric Inst.
Ferry, R. M.	Harvard Univ.
Fevold, Harry L.	Harvard Univ.
Field, John, II.	Stanford Univ.
Figge, F. H. J.	Yale Univ.
Fine, Jacob	Beth Israel Hosp., Boston
Fine, M. S.	General Foods, Inc., Hoboken, N. J.
Finland, M.	Boston City Hosp.
Firor, W. M.	Johns Hopkins Univ.
Fischer, Albert	Copenhagen, Denmark
Fischer, E.	Med. Coll. of Va.
Fischer, Martin H.	Univ. of Cincinnati
Fishberg, Ella H.	Beth Israel Hosp., N. Y.
Fitz, Reginald	Peter Bent Brigham Hosp.

Fleisher, Moyer S.	St. Louis Univ.
Florence, Laura	N. Y. Homeopathic Med.
Fluhmann, Charles F.	Stanford Univ. Med.
Foley, James O.	Univ. of Alabama
Forbes, Henry	Milton, Mass.
Forbes, John C.	Med. Coll. of Va.
Forkner, Claude E.	N. Y. City
Fortuyn, A. B. D.	Peiping Union Med. Coll.
Foster, R. H. K.	Nutley, N. J.
Fowler, Willis M.	State Univ. of Iowa
Fox, D. L.	Scripps Inst. of Oceanography
Francis, Thomas, Jr.	New York Univ. Med.
Frank, Robert T.	Mt. Sinai Hosp.
Franke, F. E.	St. Louis Univ.
Fraps, R. M.	U. S. Dept. Agri.
Frazier, Chester N.	Peiping Union Med. Coll.
Freedlander, S. O.	Western Reserve Univ.
Freedman, Louis	N. Y. City
Freeman, R. G., Jr.	Boston, Mass.
Freudenberger, C. B.	Univ. of Utah
Freund, Jules	Otisville, N. Y.
Fridericia, L. S.	Univ. of Copenhagen, Denmark
Friedemann, T. E.	Chicago, Ill.
Friedman, M. H.	Washington, D.C.
Frobisher, Martin, Jr.	Baltimore, Md.
Fulton, John F.	Yale Med.
Funk, Casimir	N. Y. City
Furth, Jacob	Cornell Univ. Med. Coll.

Gaebler, O. H.	Henry Ford Hosp., Detroit
Gager, C. Stuart	Brooklyn Botanic Garden, N. Y.
Gallagher, T. F.	Univ. of Chicago
Gamble, James L.	Harvard Univ.
Garbat, Abraham L.	Lenox Hill Hosp., N. Y.
Gardner, Leroy U.	Saranac Lab. Tuberculosis
Gardner, William U.	Yale Univ.
Garrey, Walter E.	Vanderbilt Univ.
Gaskell, John F.	Cambridge, Eng.
Gasser, Herbert S.	Rockefeller Inst.
Gaunt, Robert	New York Univ.
Geiger, E.	Inglewood, Calif.
Geiling, E. M. K.	Univ. of Chicago
Geist, Samuel H.	Mt. Sinai Hosp., N. Y. City
Gelarie, Arnold J.	N. Y. City
Gellhorn, Ernst	Univ. of Illinois Med. Coll.
Gengerelli, J. A.	Univ. of Calif., L. A.
Gerard, R. W.	Univ. of Chicago
Gerstenberger, H. J.	Western Reserve Univ.
Gesell, Robert A.	Univ. of Mich.
Gettler, A. O.	N. Y. Univ. Med. Coll.

Geyelin, H. R.	Columbia Univ.
Gibbs, O. S.	Highlands, N. C.
Gibson, R. B.	State Univ. of Iowa
Gies, William J.	Columbia Univ.
Gilechrist, Francis G.	Riverside, Calif.
Gilligan, Dorothy R.	Beth Israel Hosp., Boston
Gilman, Alfred	Yale Univ.
Gilson, A. S., Jr.	Washington Univ. Med.
Girden, Edward	Brooklyn Coll.
Githens, T. S.	Mulford Co., Philadelphia, Pa.
Givens, Maurice H.	Northwestern Yeast Co., Chicago
Glaser, Otto C.	Amherst Coll.
Glasser, Otto	Cleveland Clinic
Glick, D.	Beth Israel Hosp., Newark
Goettsch, E.	Columbia Univ.
Gold, Harry	Cornell Univ. Med. Coll.
Goldberg, S. A.	Presbyterian Hosp., Newark, N. J.
Goldblatt, Harry	Western Reserve Univ.
Goldfarb, Walter	Bellevue Hosp., N. Y.
Goldfeder, Anna	New York Univ.
Goldforb, A. J.	Coll. City of New York
Goldring, W.	N. Y. Univ. Med.
Goldschmidt, Richard	Univ. of Calif.
Goldschmidt, Samuel	Univ. of Pa.
Goodman, L.	Yale Univ.
Goodner, Kenneth	Rockefeller Inst.
Gordon, W. G.	Penn. State Coll.
Gortner, R. A.	Univ. of Minn.
Goss, C. M.	Tuscaloosa, Ala.
Goss, Harold	Univ. of Calif.
Grace, A. W.	Cornell Med. Coll.
Graeser, James B.	Univ. of Calif.
Graham, Evarts A.	Washington Univ.
Graham, Helen T.	Washington Univ.
Graves, William W.	St. Louis Univ.
Gray, H.	Stanford Hosp.
Gray, Samuel H.	Jewish Hosp., St. Louis, Mo.
Greeley, P. O.	Univ. of S. Calif.
Green, Harold D.	Western Reserve Univ.
Green, Robert G.	Univ. of Minn.
Greenberg, David M.	Univ. of Calif.
Greene, Carl H.	Brooklyn, N. Y.
Greene, Harry S. N.	Rockefeller Inst., Princeton
Greene, James A.	State Univ. of Iowa
Greenwald, Isidor	N. Y. Univ. Med. Coll.
Greenwood, Alan	Univ. of Edinburgh
Greep, R. O.	Squibb Inst., New Brunswick, N. J.
Gregersen, Magnus I.	Coll. of Phys. and Surg., N. Y.
Gregg, D. E.	Western Reserve Univ.
Gregory, Louise H.	Barnard Coll., Columbia Univ.

Gregory, Paul W.	Univ. of Calif.
Greisheimer, Esther M.	Woman's Med. Coll., Philadelphia, Pa.
Griffith, Fred R., Jr.	Univ. of Buffalo
Griffith, John Q., Jr.	Univ. of Pa.
Griffith, Wendell H.	St. Louis Univ.
Gross, Erwin G.	State Univ. of Iowa
Gross, Paul	W. Penn. Hosp., Pittsburgh
Gruber, Charles M.	Jefferson Med. Coll.
Guberlet, J. E.	Univ. of Washington
Guerrant, N. B.	Penn. State Coll.
Gunn, Francis D.	Northwestern Univ. Med.
Gurchot, Charles	Univ. of Calif. Med.
Gustavson, R. G.	Univ. of Colo.
Gustus, E. L.	Chicago, Ill.
Guthrie, C. C.	Univ. of Pittsburgh
Gutman, Alexander B.	Presbyterian Hosp., N. Y.
Guyer, Michael F.	Univ. of Wisconsin
György, Paul	Western Reserve Univ.

<b>H</b> aag, Harvey B.	Med. Coll. of Va.
Haden, Russell L.	Cleveland Clinic
Hadley, Philip	W. Penn. Hosp., Pittsburgh
Hafkesbring, H. Roberta	Woman's Med. Coll., Philadelphia
Hagan, William Arthur	Cornell Univ.
Haig, Charles	Research Div. for Chronic Diseases, N. Y.
Halberstaedter, L.	Hebrew Univ., Jerusalem
Hale, William M.	State Univ. of Iowa
Hall, Ernest M.	Univ. of S. Calif. Med.
Hall, I. C.	Univ. of Colo.
Hall, R. P.	New York Univ.
Hall, V. E.	Stanford Univ.
Halpert, Béla	Louisiana State Univ.
Halsey, John	Tulane Univ.
Halsey, Robert H.	N. Y. Post-Graduate Med.
Hambourger, Walter E.	Western Reserve Univ.
Hamilton, B. K.	Chicago, Ill.
Hammer, B. W.	Iowa State Coll.
Hampel, C. W.	American Univ., Beirut
Haney, H. F.	Univ. of Oregon Med.
Hanger, Franklin M.	Presbyterian Hosp., N. Y. City
Hanke, Martin E.	Univ. of Chicago
Hanks, J. H.	Culion, Philippine Islands
Hannon, R. R.	Cornell Med. Coll.
Hansen, Arild E.	Univ. of Minn.
Hansmann, G. H.	Columbia Hosp., Milwaukee, Wis.
Hanzal, R. F.	Western Reserve Univ.
Hanzlik, P. J.	Stanford Univ. Med.
Harde, E.	Pasteur Inst., Paris
Hardy, J. D.	Cornell Med. Coll.
Harkavy, Joseph	Mt. Sinai Hosp., N. Y.

Harkins, Henry N.....	Henry Ford Hosp., Detroit
Harned, Ben K.....	Woman's Med. Coll., Philadelphia
Harris, Isaac F.....	Tuckahoe, N. Y.
Harris, Meyer M.....	N. Y. State Psychiatric Inst.
Harris, William H.....	Tulane Univ.
Harrison, R. G. ....	Yale Univ.
Harrison, Roland W.....	Univ. of Chicago
Harrop, George A., Jr.....	New Brunswick, N. J.
Harrow, Benjamin .....	Coll. City of New York
Hart, Edwin B.....	Univ. of Wis.
Hart, George H.....	Univ. Farm, Davis, Calif.
Hartman, F. A. ....	Ohio State Univ.
Hartman, F. W.....	Ford Hospital, Detroit
Harvey, E. Newton.....	Princeton Univ.
Harvey, Samuel C.....	Yale Univ.
Hastings, A. Baird.....	Harvard Univ.
Hastings, E. G.....	Univ. of Wisc.
Haterius, H. O.....	Wayne Univ.
Hathaway, Edward S.....	Tulane Univ.
Haury, V. G.....	Jefferson Med. Coll.
Hawk, P. B.....	N. Y. City
Hayden, Charles E.....	Cornell Univ.
Hayman, J. M., Jr.....	Western Reserve Univ.
Haythorn, Samuel R.....	Singer Research Lab., Pittsburgh, Pa.
Heft, Hattie L.....	Columbia Univ.
Hegnauer, Albert H.....	Syracuse Univ.
Heidelberger, Michael.....	Presbyterian Hosp., N. Y.
Heilbrunn, L. V.....	Univ. of Pa.
Heiman, J.....	Columbia Univ.
Heinbecker, Peter .....	Washington Univ.
Helff, O. M.....	New York Univ.
Hellbaum, Arthur A.....	Univ. of Okla.
Helmer, O. M.....	Indiana Univ.
Helmholz, Henry F.....	Mayo Clinic
Hemingway, A. ....	Univ. of Minn.
Hendrix, B. M.....	Univ. of Texas
Henrici, Arthur T.....	Univ. of Minn.
Henshaw, P. S.....	Nat. Cancer Inst., Bethesda, Md.
Hepler, Opal E.....	Northwestern Univ. Med.
Herrmann, G. R.....	John Sealy Hosp., Galveston, Texas
Herrold, Russell D.....	Univ. of Ill. Med.
Hertzman, A. B.....	St. Louis Univ.
Herwick, Robert P.....	Georgetown Univ.
Hess, Walter C.....	Georgetown Univ.
Hess, Walter N.....	Hamilton Coll.
Hesseltine, H. C.....	Univ. of Chicago
Hewitt, E. A.....	Iowa State Coll.
Heymann, Walter .....	Western Reserve Univ.
Heymans, J. C.....	U. de Gand, Belgium
Hiestand, W. A.....	Purdue Univ.



Hill, F. C.	Creighton Univ. Med.
Hill, Robert M.	Univ. of Colo.
Himwich, H. E.	Albany Med. Coll.
Hines, H. M.	State Univ. of Iowa
Hinman, E. H.	Wilson Dam, Ala.
Hinman, Frank	Univ. of Calif. Med.
Hinrichs, Marie Agnes	Carbondale, Ill.
Hinsey, J. C.	Cornell Med. Coll.
Hinsshaw, H. C.	Mayo Clinic
Hirschfelder, Arthur	Univ. of Minn.
Hisaw, F. L.	Harvard Univ.
Hitchcock, Fred A.	Ohio State Univ.
Höber, Rudolf	Univ. of Pa.
Hobmaier, Michael	Univ. of Calif.
Hodge, H. C.	Univ. of Rochester
Hoeppli, R. J. C.	Peiping Union Med. Coll.
Hoffman, W. S.	Chicago Med.
Hogan, Albert G.	Univ. of Mo.
Holck, H. G. O.	Univ. of Nebraska
Hollander, Franklin	Mt. Sinai Hosp., N. Y.
Holm, George E.	Dept. Agriculture, Washington, D. C.
Holman, Emil	Stanford Univ.
Holman, R. L.	Univ. of N. Carolina
Holman, W. L.	Univ. of Toronto
Holt, L. Emmett, Jr.	Johns Hopkins Univ.
Hooker, Davenport	Univ. of Pittsburgh
Hooker, Sanford B.	Boston Univ.
Hopkins, J. Gardner	Columbia Univ.
Horsfall, F. L., Jr.	Rockefeller Foundation
Horsley, J. Shelton	St. Elizabeth's Hosp., Richmond, Va.
Horwitt, M. K.	Elgin, Ill.
Hoskins, William M.	Univ. of Calif.
Hou, Hsiang-Chuan	Lester Inst. of Med. Research, Shanghai
Howe, H. A.	Johns Hopkins Univ. Med.
Howe, Paul E.	Dept. Agriculture, Washington, D. C.
Howell, K. M.	Michael Reese Hosp., Chicago
Howitt, Beatrice F.	Univ. of Calif.
Hoyt, Anson P. S.	Univ. of S. Calif.
Hu, C. H.	Peiping Union Med. Coll.
Hu, C. K.	Peiping Union Med. Coll.
Hubbard, Roger S.	Buffalo Gen. Hosp.
Huddleston, Ora L.	Univ. of Colo.
Hudson, N. Paul	Ohio State Univ.
Hughes, Thomas P.	Uganda, E. Africa
Humphrey, R. R.	Univ. of Buffalo
Hunscher, H. A.	Western Reserve Univ.
Hunt, Reid	Harvard Univ.
Huntoon, F. M.	New Haven, Conn.
Hussey, Raymond	Baltimore, Md.

Ingersoll, E. H.....Med. Coll. of Va.  
 Ingram, Walter R.....State Univ. of Iowa  
 Irwin, M. R.....Univ. of Wise.  
 Irwin, Marion.....New York City  
 Isaacs, Raphael .....Chicago, Ill.  
 Ivy, Andrew C.....Northwestern Univ.

Jackson, C. M.....Univ. of Minn.  
 Jackson, D. E.....Univ. of Cincinnati  
 Jackson, Richard W.....U. S. Dept. of Agriculture  
 Jacobs, Henry R.....Rockefeller Foundation  
 Jacobs, J. L.....Tufts Coll.  
 Jacobs, M. H.....Univ. of Pa.  
 Jacobs, Walter A.....Rockefeller Inst., N. Y.  
 Jacobsen, Victor C.....Troy, N. Y.  
 Jaffe, Henry L.....Hosp. for Joint Diseases, N. Y.  
 Jahn, Theodore L.....State Univ. of Iowa  
 Jameson, Eloise.....Stanford Univ.  
 Jeans, Philip C.....Univ. of Iowa  
 Jennings, H. S.....Univ. of California, Los Angeles  
 Jensen, Hans F.....Squibb Inst., New Brunswick, N. J.  
 Jobling, J. W.....Columbia Univ.  
 Johlin, J. M.....Vanderbilt Univ. Med.  
 Johnson, C. A.....Univ. of Chicago  
 Johnson, C. C.....Univ. of Utah  
 Johnson, F. H.....Princeton Univ.  
 Johnson, T. B.....Yale Univ.  
 Johnston, C. G.....Wayne Univ. Med.  
 Jolliffe, Norman H.....N. Y. Univ. Med. Coll.  
 Jonas, Leon .....Univ. of Pa.  
 Jones, David B.....U. S. Dept. of Agri.  
 Jones, Kenneth K.....Northwestern Univ.  
 Jones, L. R.....St. Louis Univ.  
 Jordan, H. E.....Univ. of Va.  
 Jorstad, L. H.....Barnard Skin and Cancer Hosp., St. Louis, Mo.  
 Joslin, E. P.....Harvard Univ.  
 Juhn, Mary .....Beltsville, Md.  
 Jukes, T. H.....Univ. of Calif.  
 Julianelle, Louis A.....Washington Univ.  
 Jung, F. T.....Northwestern Univ. Med.  
 Jungeblut, Claus .....Coll. of Phys. and Surg., N. Y.

Kahn, Morton C.....Cornell Med. Coll., N. Y.  
 Kahn, R. L.....Univ. Hosp., Ann Arbor, Mich.  
 Kamm, Oliver .....Parke, Davis & Co., Detroit  
 Kaplan, Alex.....Univ. of Calif.  
 Karelitz, Samuel.....Mt. Sinai Hosp., N. Y.  
 Karsner, H. T.....Western Reserve Univ.  
 Kast, Ludwig.....N. Y. Post-Graduate Med.  
 Katz, Gerhard .....Tulane Univ.

Katz, L. N.....	Michael Reese Hosp., Chicago
Katzenelbogen, S.....	Washington, D. C.
Katzman, Philip A.....	St. Louis Univ.
Keefer, Chester S.....	Boston, Mass.
Keeton, Robert W.....	Univ. of Ill.
Keiles, Elsa Orent.....	U. S. Dept. Agri.
Keitt, G. W.....	Univ. of Wise.
Keller, Allen D.....	Univ. of Alabama Med.
Kelly, G. L.....	Univ. of Georgia Med.
Kemp, Hardy A.....	Univ. of Vermont
Kemp, Jarold E.....	Johns Hopkins Univ.
Kendall, Arthur I.....	Northwestern Univ. Med.
Kendall, E. C.....	Mayo Clinic, Minn.
Kendall, Forrest E.....	Douglaston, L. I.
Kerr, William J.....	Univ. of Calif. Med.
Kessel, John F.....	Univ. of S. Calif.
Kesten, H. D.....	Coll. of Phys. and Surg., N. Y.
Key, John A.....	Washington Univ.
Keys, Ancel.....	Univ. of Minn.
Kidd, John G.....	Rockefeller Inst.
Killian, J. A.....	N. Y. Post-Graduate Med.
Kindred, J. E.....	Univ. of Va.
King, Charles G.....	Univ. of Pittsburgh
King, Helen D.....	Wistar Inst., Philadelphia
King, Joseph T.....	Univ. of Minn.
Kinsella, Ralph A.....	St. Louis Univ.
Kirkbride, Mary B.....	State Dept. of Health, Albany, N. Y.
Kissin, Milton.....	Beth Israel Hosp., N. Y.
Kleiber, Max.....	Univ. of Calif., Davis
Kleiner, I. S.....	N. Y. Med. Coll.
Kleitman, Nathaniel.....	Univ. of Chicago
Kligler, I. J.....	Hebrew Univ., Palestine
Kline, B. S.....	Mt. Sinai Hosp., Cleveland
Klüver, H.....	Univ. of Chicago
Knoefel, P. K.....	Univ. of Louisville
Knowlton, Frank P.....	Syracuse Univ.
Knowlton, G. C.....	Cornell Med. Coll.
Knudson, Arthur.....	Albany Med. Coll.
Kober, Philip A.....	Detroit, Mich.
Koch, Fred C.....	Univ. of Chicago
Koehler, Alfred E.....	Santa Barbara Hosp., Calif.
Kofoed, Charles A.....	Univ. of Calif.
Kohn, Jerome L.....	Mt. Sinai Hosp., N. Y.
Kohn-Richards, R.....	Abbott Lab., Chicago
Kolmer, John A.....	Univ. of Pa.
Kopeloff, Nicholas.....	Psychiatric Inst., N. Y.
Koppanyi, Theodore.....	Georgetown Univ. Med.
Korns, Horace M.....	State Univ. of Iowa
Koser, Stewart.....	Univ. of Chicago
Koster, Harry.....	Crown Heights Hosp., Brooklyn

Kramer, Benjamin.....	Brooklyn Jewish Hosp.
Kramer, S. D.....	Dept. of Health, Lansing, Mich.
Krantz, J. C., Jr.....	Univ. of Maryland Med.
Krasnow, Frances .....	Guggenheim Dental Clinic, N. Y.
Kriss, M.....	Pennsylvania State Coll.
Krizenecky, J.....	Brno, Czechoslovakia
Kronfeld, P. C.....	Peiping Union Med. Coll.
Krueger, A. P.....	Univ. of Calif.
Krumbhaar, E. B.....	Univ. of Pa.
Kruse, Theophile K.....	Univ. of Pittsburgh
Kugelmass, I. Newton.....	N. Y. City
Kunde, Margaret M.....	Chicago, Ill.
Kunitz, Moses.....	Rockefeller Inst., Princeton, N. J.
Kuntz, Albert.....	St. Louis Univ.
Kurotekin, T. J.....	Lederle Lab.
Kurzrok, Raphael .....	Coll. of Phys. and Surg., N. Y.
Kuttner, Ann G.....	Irvington, N. Y.
<b>L</b>	
Ladd, William S.....	Cornell Univ. Med. Coll.
Lambert, R. A.....	Rockefeller Foundation
Lamson, Paul D.....	Vanderbilt Univ.
Lamson, Robert W.....	Los Angeles, Calif.
Lancefield, D. E.....	Queens Coll., Flushing, N. Y.
Landis, Carney.....	Psychiatric Inst., N. Y.
Landis, E. M.....	Univ. of Va.
Lands, A. M.....	Georgetown Univ. Med.
Landsteiner, Karl .....	Rockefeller Inst., N. Y.
Langstroth, Lovell.....	San Francisco, Calif.
Larkum, N. W.....	Charlottesville, Va.
Larsell, Olaf.....	Univ. of Oregon
Larson, C. E.....	College of the Pacific
Larson, Edward.....	Temple Univ.
Larson, John A.....	Detroit, Mich.
Larson, P. S.....	Detroit, Mich.
Larson, W. P.....	Univ. of Minn.
Laurens, Henry .....	Tulane Univ.
Lawrence, John H. ....	Univ. of Calif.
Lawson, Hampden C.....	Univ. of Louisville
Leake, C. D.....	Univ. of Calif. Med.
Leake, J. P.....	Hygienic Lab., Washington, D. C.
Lee, Ferdinand S.....	Johns Hopkins Med.
Lee, Milton O.....	Harvard Univ.
Leese, Chester E.....	George Washington Univ.
Lehman, A. J.....	Wayne Univ. Med.
Leiter, Louis.....	Univ. of Chicago
Lennette, E. H.....	Rockefeller Inst.
Leonard, C. S.....	Univ. of Vermont
Leonard, S. L.....	Rutgers Univ.
Leonard, Veader.....	Johns Hopkins Univ.
Levin, Louis .....	Coll. of Phys. and Surg., N. Y.

Levine, Benjamin S.	Arlington, Va.
Levine, Max	Iowa State Coll.
Levine, Michael	Montefiore Hosp., N. Y. City
Levine, Philip	Beth Israel Hosp., Newark, N. J.
Levine, Samuel Z.	Cornell Univ. Med. Coll.
Levine, Victor E.	Creighton Univ.
Levinson, Samuel A.	Univ. of Ill.
Levy, Milton	N. Y. Univ. Med. Coll.
Levy, Robert L.	Presbyterian Hosp., N. Y. City
Lewis, Howard B.	Univ. of Mich.
Lewis, Robert C.	Univ. of Colo.
Lewisohn, R.	Mt. Sinai Hosp., N. Y.
Li, R. C.	Peiping Union Med. Coll.
Lichtenstein, Louis	Hosp. for Joint Diseases, N. Y. City
Liddell, H. S.	Cornell Univ.
Lieb, C. C.	Columbia Univ.
Lillie, Frank R.	Univ. of Chicago
Lillie, Ralph S.	Univ. of Chicago
Lim, C. E.	Peiping Union Med. Coll.
Lim, Robert K.	Peiping Union Med. Coll.
Lindegren, C. C.	Univ. of S. Calif.
Lindsley, Donald B.	Brown Univ.
Linegar, C. R.	Georgetown Univ. Med.
Linton, Richard W.	Cornell Med. Coll.
Lipman, Charles B.	Univ. of California
Little, C. C.	Bar Harbor, Me.
Liu, Shih-hao	Peiping Union Med. Coll.
Liu, Szu-chih	Peiping Union Med. Coll.
Locke, A. P.	W. Pennsylvania Hosp., Pittsburgh
Loeb, Leo	Washington Univ.
Loeb, Robert F.	Presbyterian Hosp., N. Y. City
Loebel, Robert O.	Cornell Med. Coll.
Loehwing, W. F.	Univ. of Iowa
Loewe, Siegfried	New Rochelle, N. Y.
London, E. S.	Leningrad, U. S. S. R.
Long, C. N. H.	Yale Univ.
Long, E. R.	Henry Phipps Inst., Philadelphia
Long, Perrin H.	Johns Hopkins Hosp.
Longcope, W. T.	Johns Hopkins Univ.
Looney, J. M.	Worcester State Hosp., Mass.
Lorenz, William F.	Univ. of Wise.
Lucas, William P.	Univ. of Calif.
Lucia, S. P.	Univ. of Calif.
Luck, J. Murray	Stanford Univ.
Lucké, Balduin	Univ. of Pa.
Luckhardt, A. B.	Univ. of Chicago
Lueth, H. C.	Chicago, Ill.
Lukens, F. D. W.	Univ. of Pa.
Lund, E. J.	Univ. of Texas
Lurie, Max B.	Henry Phipps Inst., Philadelphia



Luyet, B. J.....	St. Louis Univ.
Lyman, J. F.....	Ohio State Univ.
Lynch, Clara J.....	Rockefeller Inst., N. Y. City
Lyons, W. R.....	Univ. of Calif.
<b>Mc</b> Cann, William S.....	Univ. of Rochester
McCaughan, J. M.....	St. Louis Univ. Med.
McClendon, J. Francis.....	Hahnemann Med. Coll.
McClintock, John T.....	Univ. of Iowa
McClung, L. S.....	Indiana Univ.
McCollum, E. V.....	Johns Hopkins Univ.
McCoy, Oliver R.....	Univ. of Rochester
McCullagh, D. R.....	Cleveland Clinic Foundation
McCutcheon, Morton.....	Univ. of Pa.
McEachern, Donald .....	Montreal Neurological Inst.
McEllroy, W. S.....	Univ. of Pittsburgh
McEwen, Currier.....	New York Univ. Med.
McGinty, Daniel A.....	Parke, Davis Co.
McGuigan, H. A.....	Univ. of Illinois Med.
McIntosh, Rustin.....	Babies Hosp., N. Y. City
McIntyre, A. R.....	Univ. of Nebraska
McIver, M. A.....	Bassett Hosp., Cooperstown, N. Y.
McKenzie, F. F.....	Univ. of Mo.
McKibben, Paul S.....	Univ. of S. Calif.
McKinley, J. C.....	Univ. of Minn.
McLean, Franklin C.....	Univ. of Chicago
McMaster, Philip D.....	Rockefeller Inst., N. Y. City
McMeans, J. W.....	Pittsburgh Hosp.
McNaught, J. B.....	Stanford Univ. Med.
McQuarrie, Irvine .....	Univ. of Minn.
Ma, W. C.....	Peiping Union Med. Coll.
MacDowell, E. Carlton.....	Exp. Evolution, Cold Spring Harbor, N. Y.
Macht, D. I.....	Hynson, Westcott and Dunning, Baltimore
MacKay, E. M.....	Scripps Metabolic Clinic, San Diego, Calif.
Mackenzie, George M.....	Bassett Hosp., Cooperstown, N. Y.
MacLeod, Grace.....	Columbia Univ.
MacNeal, Ward J.....	N. Y. Post-Graduate Med.
MacNider, William de B.....	Univ. of N. Carolina
Maey, I. G.....	Detroit, Mich.
Magath, T. B.....	Mayo Clinic
Magill, T. P.....	Cornell Med. Coll.
Main, Roland J.....	Med. Coll. of Va.
Maison, George L.....	Wayne Univ.
Malamud, William .....	Worcester, Mass.
Mallman, W. Leroy.....	Michigan State Coll.
Mallory, Frank .....	Boston City Hosp.
Maloney, A. H.....	Howard Univ.
Maltaner, E. J.....	N. Y. State Dept. of Health, Albany
Maltaner, Frank.....	N. Y. State Dept. of Health, Albany
Mann, Frank C.....	Mayo Clinic

Mann, Hubert .....	N. Y. City
Manville, Ira Albert.....	Univ. of Oregon
Manwaring, W. H.....	Stanford Univ.
Manwell, Reginald D.....	Syracuse Univ.
Marine, David.....	Montefiore Hosp., N. Y.
Marmorsten, J.....	New York City
Marsh, R. P.....	New Brunswick, N. J.
Marshall, E. K., Jr.....	Johns Hopkins Univ.
Marshall, Max S.....	Univ. of Calif.
Marshall, Wade H.....	Johns Hopkins Univ.
Martin, Lay.....	Johns Hopkins Univ.
Martin, S. J.....	Bellevue Hosp., N. Y.
Mason, K. E.....	Univ. of Rochester
Master, Arthur M.....	N. Y. City
Matas, Rudolph.....	New Orleans, La.
Mattill, Henry A.....	State Univ. of Iowa
Maughan, G. H.....	Etna, N. Y.
Maver, Mary E.....	National Inst. of Health, Washington
Maxey, Kenneth F.....	Johns Hopkins Univ.
Mayerson, H. S.....	Tulane Univ.
Maynard, L. A.....	Cornell Univ.
Meakins, Jonathan.....	McGill Univ.
Medes, Grace.....	Lankenau Research Inst., Philadelphia
Medlar, E. M.....	Mt. McGregor, N. Y.
Meek, Walter J.....	Univ. of Wise.
Megrail, Emerson.....	Western Reserve Univ.
Meigs, E. B.....	Dairy Division Exp. Station, Beltsville, Md.
Mellon, Ralph R.....	W. Penn. Hosp., Pittsburgh
Menkin, Valy.....	Harvard Med.
Menten, Maude L.....	Univ. of Pittsburgh
Menville, Leon J.....	Tulane Univ.
Meranze, D. R.....	Jefferson Med. Coll.
Mettier, Stacy R.....	Univ. of Calif. Med.
Mettler, F. A.....	Univ. of Georgia Med.
Meyer, Adolf.....	Johns Hopkins Univ.
Meyer, A. E.....	N. Y. City
Meyer, A. W.....	Stanford Univ. Med.
Meyer, Karl.....	Coll. of Phys. and Surg., N. Y.
Meyer, K. F.....	Univ. of Calif.
Meyer, O. O.....	Univ. of Wise.
Meyer, R. K.....	Univ. of Wise.
Michaelis, Leonor.....	Rockefeller Inst.
Michels, N. A.....	Jefferson Med.
Middleton, W. S.....	Univ. of Wise.
Miles, W. R.....	Yale Univ.
Milhorat, A. T.....	Cornell Med. Coll.
Miller, C. Phillip, Jr.....	Univ. of Chicago
Miller, E. G., Jr.....	Coll. of Phys. and Surg., N. Y.
Miller, E. S.....	Univ. of Minn.
Miller, Franklin R.....	Jefferson Med. Coll.

Miller, Frederick R.	Univ. of W. Ontario
Miller, G. H.	Amer. Univ. of Beirut, Syria
Millet, John A. P.	N. Y. City
Minot, George R.	Boston City Hosp.
Mirsky, Alfred E.	Rockefeller Inst.
Mirsky, I. Arthur	Jewish Hosp., Cincinnati
Mitchell, Helen S.	Mass. State Coll.
Mitchell, O. W. H.	Syracuse Univ.
Molitor, H.	Rahway, N. J.
Montgomery, M. L.	Univ. of California
Moon, V. H.	Jefferson Med. Coll.
Moore, A. R.	Univ. of Oregon
Moore, Carl R.	Univ. of Chicago
Moore, M. M.	Univ. of Oregon
Moore, R. A.	Washington Univ. Med.
Morales-Otero, P.	School of Tropical Med., Puerto Rico
Morgan, A. F.	Univ. of California
Morgan, T. H.	Calif. Inst. of Technology
Moritz, A. R.	Harvard Med.
Morrell, J. A.	E. R. Squibb and Sons
Morse, Arthur	New Haven Hosp.
Morton, John J.	Univ. of Rochester Med.
Mosenthal, H. O.	N. Y. Post-Graduate Med.
Mu, J. W.	Peiping Union Med. Coll.
Muckenfuss, R. S.	N. Y. City Bd. of Health
Mudd, Stuart	Univ. of Pennsylvania
Mudge, C. S.	Univ. of Calif.
Muehlberger, C. W.	Northwestern Univ.
Mueller, J. Howard	Harvard Univ.
Mulinos, M. G.	Coll. of Phys. and Surg., N. Y.
Mull, J. W.	Western Reserve Univ.
Muller, G. L.	Cambridge, Mass.
Muller, H. J.	Edinburgh, Scotland
Mullin, Francis J.	Univ. of Chicago
Muntwyler, Edward	Western Reserve Univ.
Murlin, John R.	Univ. of Rochester
Murphy, D. P.	Univ. of Pennsylvania
Murphy, J. B.	Rockefeller Inst.
Murray, Thomas J.	Rutgers Univ.
Musser, John H.	Tulane Univ.
Myers, Chester N.	Columbia Univ.
Myers, Victor C.	Western Reserve Univ.
<b>N</b> adler, J. Ernest	New York Univ. Med.
Nadler, Samuel B.	Tulane Univ.
Nathanson, I. T.	Harvard Univ.
Nathanson, Morris H.	Los Angeles, Calif.
Naumann, H. N.	Brooklyn, N. Y.
Necheles, H.	Michael Reese Hosp., Chicago
Nedzel, A. J.	Univ. of Illinois Med.

Neill, J. M.	Cornell Med. Coll.
Nelson, Elmer M.	U. S. Dept. of Agriculture
Nelson, Erwin E.	Tulane Univ.
Nelson, Thurlow C.	Rutgers Univ.
Nelson, Victor E.	Iowa State Coll.
Nelson, W. O.	Wayne Univ.
Neter, Erwin	Univ. of Buffalo
Neuwirth, Isaac	New York Univ.
Newburgh, Louis H.	Univ. of Michigan
Newman, Henry W.	Stanford Univ. Med.
Ni, Tsang-gi	Lester Inst., Shanghai, China
Nice, L. B.	Chicago, Ill.
Nicholas, J. S.	Yale Univ.
Nicholls, Edith E.	Danville, Pa.
Nichols, M. S.	Univ. of Wisc.
Nicholson, Hayden C.	Univ. of Mich.
Nielson, Carl	Abbott Laboratories, Chicago
Niles, Walter L.	Cornell Med. Coll.
Norris, Earl R.	Univ. of Wash.
Norris, L. C.	Cornell Univ.
Northrop, John H.	Rockefeller Inst., Princeton, N. J.
Norton, J. F.	Upjohn Co., Kalamazoo, Mich.
Novy, Frederick G.	Univ. of Mich.
Nungester, W. J.	Univ. of Mich.
Nye, R. N.	Boston City Hosp.
<b>O</b> brechkove, Vasil	Bard Coll.
Ochsner, E. W. A.	Tulane Univ.
Oertel, Horst	McGill Univ.
Oesting, R. B.	Chicago, Ill.
Ogden, Eric	Univ. of Calif.
Okey, Ruth E.	Univ. of Calif.
Oldberg, Eric	Univ. of Ill. Med.
O'Leary, J. L.	Washington Univ.
Olitsky, Peter K.	Rockefeller Inst., N. Y. City
Oliver, Jean	Long Island Coll., Brooklyn
Oliver, W. W.	Long Island Coll. of Med.
Olmstead, Miriam P.	Presbyterian Hosp., N. Y. City
Olmsted, J. M. D.	Univ. of Calif.
Olmsted, W. H.	Washington Univ.
Opie, Eugene L.	Cornell Univ. Med. Coll.
Oppenheimer, B. S.	Columbia Univ.
Ornstein, George G.	Columbia Univ.
Orten, James M.	Wayne Univ.
Osgood, E. E.	Univ. of Oregon Med.
Osterhout, W. J. V.	Rockefeller Inst.
Ottenberg, R.	Mt. Sinai Hosp., N. Y.
Oughterson, A. W.	Yale Univ.

Pack, George T.	Memorial Hosp., N. Y.
Page, E. W.	Univ. of Calif.
Page, Irvine H.	Indianapolis, Ind.
Pak, Chubyung	Lester Inst. Med. Research, Shanghai
Palmer, Leroy S.	Univ. of Minn.
Palmer, W. L.	Univ. of Chicago
Palmer, W. W.	Presbyterian Hosp., N. Y.
Papanicolaou, George N.	Cornell Univ. Med. Coll.
Pappenheimer, A. M.	Coll. of Phys. and Surg., N. Y.
Pappenheimer, A. M., Jr.	Univ. of Penn.
Park, E. A.	Johns Hopkins Univ.
Parker, Frederick, Jr.	Boston City Hosp.
Parker, George H.	Harvard Univ.
Parr, L. W.	George Washington Univ.
Parsons, Helen T.	Univ. of Wise.
Paschkis, K. E.	Jefferson Med. Coll.
Patterson, Thomas L.	Wayne Univ.
Paul, William D.	State Univ. of Iowa
Pearce, Louise	Rockefeller Inst.
Pearse, Herman E.	Univ. of Rochester
Pearson, Paul B.	Agri. & Mech. Coll., Texas
Pease, Marshall C.	N. Y. Post-Graduate Med.
Peek, Samuel M.	Mt. Sinai Hosp., N. Y.
Pellini, Emil J.	N. Y. Univ. Med. Coll.
Pemberton, Ralph	Memorial Hosp., Abington, Pa.
Pencharz, R. I.	Kentfield, Calif.
Penfield, Wilder G.	McGill Univ.
Pepper, O. H. Perry	Univ. of Pa.
Perlzweig, William A.	Duke Univ.
Permar, Howard H.	Mercy Hosp., Pittsburgh
Perry, Isabella H.	Univ. of Calif.
Peskind, Samuel	Cleveland, O.
Peters, John P.	Yale Univ.
Petersen, W. E.	Univ. of Minn.
Peterson, W. H.	Univ. of Wisconsin
Petroff, S. A.	Seaview Hosp., Staten Island
Pett, L. B.	Univ. of Alberta, Canada
Pfeiffer, C. A.	Yale Univ.
Pfeiffer, C. C.	Wayne Univ.
Pfeiffer, J. A. F.	Johns Hopkins Univ.
Pfiffner, J. J.	Parke, Davis and Co., Detroit
Phemister, D. B.	Univ. of Chicago
Phillips, P. H.	Univ. of Wise.
Phillips, Robert A.	Cornell Med. Coll.
Pick, E. P.	N. Y. City
Pierce, Harold B.	Univ. of Vermont
Pilot, I.	Univ. of Ill. Med.
Pincussen, L.	Univ. of Ill. Med.
Pinkerton, Henry	St. Louis, Mo.
Pinkston, J. O.	American Univ., Beirut



Pinner, Max.....	Bedford Hills, N. Y.
Pittman, Margaret.....	Nat. Inst. of Health, Bethesda, Md.
Plass, E. D.....	Univ. of Iowa
Plotz, Harry .....	Army Medical School
Poe, Charles F.....	Univ. of Colo.
Pohlman, Augustus G.....	Los Angeles, Calif.
Pollack, H.....	Cornell Univ. Med.
Pollock, L. F.....	Northwestern Univ.
Popper, H. L.....	Michael Reese Hosp., Chicago
Ponder, Eric.....	Cold Spring Harbor, N. Y.
Powell, H. M.....	Eli Lilly and Co., Indianapolis
Powers, Grover F.....	Yale Univ.
Pratt, Frederick H.....	Boston Univ.
Pratt, Joseph H.....	Tufts Med.
Pribram, Ernest.....	Chicago, Ill.
Proescher, F.....	San José, Calif.
Puestow, C. B.....	Univ. of Ill. Med.

Quick, Armand J.....	Milwaukee, Wis.
Quigley, J. P.....	Western Reserve Med.
Quinby, W. C.....	Peter Bent Brigham Hosp.

Ragins, Ida Kraus.....	Univ. of Chicago
Rahn, O.....	Cornell Univ.
Raiziss, George W.....	Inst. Cutaneous Med., Philadelphia
Rake, Geoffrey W.....	New Brunswick, N. J.
Rakieten, M. L.....	Long Island Coll. of Med.
Ralli, Elaine P.....	N. Y. Univ. Med. Coll.
Ramaley, Francis.....	Univ. of Colo.
Ranson, S. W.....	Northwestern Univ.
Rapport, David .....	Tufts Coll. Med.
Rasmussen, A. T.....	Univ. of Minn.
Ratner, Bret .....	N. Y. Univ. Med. Coll.
Ravdin, I. S.....	Univ. of Pa.
Ray, G. B.....	Long Island Coll. of Med.
Ray, Henry M.....	S. Side Hosp., Pittsburgh
Read, Bernard E.....	Lester Inst., Shanghai, China
Read, J. Marion.....	Stanford Univ. Med.
Reece, R. P.....	Rutgers Coll.
Reed, Carlos I.....	Univ. of Ill. Med.
Reichert, F. L.....	Stanford Univ.
Reimann, Hobart A.....	Jefferson Med. Coll.
Reimann, Stanley P.....	Univ. of Pa.
Reiner, L.....	Burroughs Wellcome and Co.
Reynolds, Chapman .....	Louisiana State Univ.
Reynolds, S. R. M.....	Long Island Coll. of Med.
Reznikoff, Paul.....	Cornell Univ. Med. Coll.
Rhoads, Cornelius P.....	Memorial Hosp., N. Y.
Rhoads, Paul S.....	Northwestern Univ.
Rieh, Arnold R.....	Johns Hopkins Univ.

Richards, Alfred N.....	Univ. of Pa.
Richards, Leah B.....	Memorial Hosp., N. Y. City
Richards, Oscar W.....	Buffalo, N. Y.
Richardson, A. P.....	Stanford Univ. Med.
Richardson, R.....	Univ. of Pa.
Richter, Maurice.....	N. Y. Post-Graduate Med.
Riddle, Oscar.....	Exp. Evolution, Cold Spring Harbor, N. Y.
Rinehart, James F.....	Univ. of Calif.
Ringer, Michael.....	Montefiore Hosp., N. Y.
Rivers, Thomas M.....	Rockefeller Inst.
Robb, Jane Sands.....	Syracuse Univ.
Roberts, Elmer.....	Univ. of Ill.
Roberts, R. G.....	Chicago Med. School
Robertson, Elizabeth C.....	Univ. of Toronto
Robertson, Oswald H.....	Univ. of Chicago
Robinson, Charles S.....	Vanderbilt Univ.
Robinson, Elliott.....	Antitoxin Laboratory, Boston
Roe, Joseph H.....	George Washington Univ.
Rogers, Fred T.....	Baylor Univ.
Rogoff, J. M.....	Univ. of Pittsburgh
Rohdenburg, George L.....	Lenox Hill Hosp., N. Y.
Romanoff, Alexis L.....	Cornell Univ.
Ronzoni, Ethel.....	Washington Univ.
Roome, N. W.....	London, Ont.
Root, W. S.....	Coll. of Phys. and Surg.
Rosahn, Paul D.....	Yale Univ.
Rose, Anton R.....	Prudential Life Insurance Co., Newark, N. J.
Rose, William C.....	Univ. of Ill.
Rosen, Samuel H.....	Montefiore Hosp., N. Y.
Rosenow, E. C.....	Mayo Foundation
Rosenthal, Lazar.....	Israel-Zion Hosp., Brooklyn, N. Y.
Rosenthal, S. R.....	Univ. of Ill. Med.
Ross, Victor.....	N. Y. City
Roth, George B.....	George Washington Univ.
Rous, Peyton.....	Rockefeller Inst.
Rovenstine, E. A.....	New York Univ. Med.
Rowntree, L. G.....	Philadelphia Inst. for Med. Research
Rubin, Morton A.....	Worcester State Hosp., Mass.
Rubinstein, Hyman.....	Mt. Sinai Hosp., Baltimore
Rulon, Olin.....	Wayne Univ.
Rugh, Roberts.....	Hunter College, N. Y.
Rusch, H. P.....	Univ. of Wisc.
Russell, W. C.....	N. J. Agr. Exp. Station
Rytand, D. A.....	Stanford Univ.
Sabin, Albert R.....	Children's Hosp., Cincinnati
Sacks, Jacob.....	Univ. of Mich.
Sahyun, Melville.....	Detroit, Mich.
Salant, William.....	N. Y. City
Salle, A. J.....	Univ. of Calif.

Salmon, U. J.	Mt. Sinai Hosp., N. Y. City
Salter, W. T.	Boston City Hosp.
Salvesen, Harold A.	Rikshospitalet, Oslo, Norway
Samuels, L. T.	Univ. of Minn.
Sandberg, M.	Montefiore Hosp., N. Y. City
Sanford, A. H.	Mayo Clinic
Sansum, W. D.	Santa Barbara Cottage Hosp., Calif.
Saunders, Felix	Scripps Institution of Oceanography
Sawyer, W. A.	Rockefeller Foundation
Sayles, L. P.	College City of N. Y.
Scammon, R. E.	Univ. of Minn.
Schattenberg, H. J.	Tulane Univ.
Schechtman, A. M.	Univ. of Calif., L. A.
Scheff, George	New Haven, Conn.
Schick, Béla	Mt. Sinai Hosp., N. Y. City
Schiff, F.	Beth Israel Hosp., N. Y. City
Schlesinger, M. J.	Beth Israel Hosp., Boston
Schloss, Oscar M.	Cornell Univ. Med. Coll.
Schlutz, F. W.	Univ. of Chicago
Schmidt, Carl F.	Univ. of Pa.
Schmidt, Carl L. A.	Univ. of Calif.
Schmitt, Francis O.	Washington Univ.
Schneider, Edward C.	Wesleyan Univ.
Schooley, J. P.	Detroit, Mich.
Schour, Isaac	Univ. of Ill. Dental Coll.
Schultz, E. W.	Stanford Univ.
Schultz, O. T.	St. Francis Hosp., Evanston, Ill.
Schwartz, Benjamin	U. S. Dept. of Agr.
Schwind, J. L.	Albany Med. Coll.
Scott, E. L.	Columbia Univ.
Scott, F. H.	Univ. of Minn.
Scott, Gordon H.	Washington Univ.
Scott, Leonard C.	Tulane Univ.
Scott, R. W.	Western Reserve Univ.
Scott, W. J. M.	Univ. of Rochester
Seager, L. D.	Univ. of Tenn.
Sears, H. J.	Univ. of Oregon
Seastone, Charles V.	Univ. of Wise.
Seecof, David P.	N. Y. City
Seegal, Beatrice	Coll. of Phys. and Surg., N. Y.
Seegal, David	Research Div. of Chronic Diseases, N. Y. City
SeEVERS, M. H.	Univ. of Wise.
Seibert, Florence B.	Henry Phipps Inst., Philadelphia
Selle, W. A.	Univ. of Texas
Selye, Hans	McGill Univ.
Sendroy, Julius, Jr.	Loyola Univ.
Severinghaus, A. E.	Coll. of Phys. and Surg., N. Y.
Severinghaus, Elmer L.	Univ. of Wise.
Shafer, George D.	Stanford Univ.
Shaffer, Philip A.	Washington Univ.

Shaklee, A. O.	St. Louis Univ.
Shannon, J. A.	N. Y. Univ. Med.
Shapiro, Herbert	Vassar Coll.
Sharlit, Herman	Roosevelt Hosp., N. Y. City
Sharp, P. F.	Cornell Univ.
Shaughnessy, H. J.	Ill. Dept. Public Health
Shaw, E. H., Jr.	Univ. of S. Dakota
Shear, M. J.	Nat. Cancer Inst., Bethesda, Md.
Sheard, Charles	Mayo Clinic
Sheinin, J. J.	Chicago Med.
Sherman, H. C.	Columbia Univ.
Sherman, Hartley E.	Bureau of Science, Manila, P. I.
Sherman, James M.	Cornell Univ.
Sherwin, Carl P.	Fordham Univ.
Shibley, Gerald S.	Cleveland, O.
Shilling, C. W.	New London, Conn.
Shinn, L. E.	W. Penn. Hosp., Pittsburgh
Shiple, George J.	Univ. of Detroit
Shock, N. W.	Univ. of Calif.
Shohl, Alfred T.	Harvard Univ.
Shope, R. E.	Rockefeller Inst., Princeton, N. J.
Shorr, E.	Coll. of Phys. and Surg.
Shwartzman, Gregory	Mt. Sinai Hosp., N. Y.
Sia, Richard H. P.	Univ. of Hawaii
Sickles, Grace M.	N. Y. State Dept. of Health
Silberberg, Martin	New York Univ. Med. Coll.
Silverman, D. N.	Tulane Univ.
Simmons, James S.	Office of Surgeon General, Washington, D. C.
Simonds, J. P.	Northwestern Univ.
Sinclair, R. G.	Queens Univ., Canada
Smadel, J. E.	Rockefeller Inst.
Smelser, G. K.	Coll. Phys. and Surg., N. Y.
Smetana, Hans	Columbia Univ.
Smith, Arthur H.	Wayne Univ.
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Smith, Clarence	Bergenfield, N. J.
Smith, Clayton S.	Ohio State Univ.
Smith, David T.	Duke Univ.
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Smith, Fred M.	Univ. of Iowa
Smith, George V.	Hosp. for Women, Brookline, Mass.
Smith, Harry P.	State Univ. of Iowa
Smith, Homer W.	N. Y. Univ. Med. Coll.
Smith, Lawrence W.	Temple Univ., Philadelphia
Smith, Maurice I.	National Inst. of Health, Washington
Smith, Millard	Boston, Mass.
Smith, Paul K.	Yale Univ.
Smith, Paul W.	Univ. of Tenn.
Smith, Philip E.	Columbia Univ.
Smithburn, K. C.	Uganda, E. Africa

Smyly, H. Jocelyn.....	Shantung Christian Univ., China
Snapper, L.....	Peiping Union Med. Coll.
Snyder, Franklin F.....	Univ. of Chicago
Sobotka, H. H.....	Mt. Sinai Hosp., N. Y.
Sodeman, William A.....	Tulane Univ.
Soffer, Louis J.....	Mt. Sinai Hosp., N. Y.
Sollmann, Torald.....	Western Reserve Univ.
Somogyi, Michael.....	Jewish Hosp., St. Louis, Mo.
Soskin, Samuel.....	Michael Reese Hosp., Chicago
Soule, M. H.....	Univ. of Mich.
Sperry, Warren.....	N. Y. State Psychiatric Inst.
Spiegel, Ernest.....	Temple Univ.
Spiegel-Adolf, Mona.....	Temple Univ.
Spies, T. D.....	Birmingham, Ala.
Spink, W. W.....	Univ. of Minn.
Sponsler, O. L.....	Univ. of Calif., L. A.
Sprunt, D. H.....	Duke Hosp., N. Carolina
Stainsby, W. J.....	Danville, Pa.
Stanley, Wendell M.....	Rockefeller Inst.
Stannard, J. N.....	Emory Univ.
Stark, Clifford N.....	Cornell Univ.
Stark, Mary B.....	New York Med. Coll.
Starr, M. P.....	Northwestern Univ. Med.
Stasney, J.....	Louisiana State Univ.
Steele, John M.....	Welfare Hosp., N. Y.
Steggerda, F. R.....	Univ. of Ill.
Steinbach, M. Maxim.....	Coll. of Phys. and Surg., N. Y.
Steinberg, Bernhard.....	Toledo Hosp., Toledo, Ohio
Stekol, J. A.....	Fordham Univ.
Stenstrom, Wilhelm.....	Univ. of Minn.
Stewart, Harold J.....	New York Hosp.
Stewart, Harold L.....	Nat. Cancer Inst., Bethesda, Md.
Still, Eugene U.....	Univ. of Chicago
Stillman, Ralph G.....	Cornell Univ.
Stockton, A. B.....	Stanford Univ. Med.
Stoesser, Albert V.....	Univ. of Minn.
Stone, Calvin P.....	Stanford Univ.
Stone, Leon S.....	Yale Univ.
Storey, Thomas A.....	Stanford Univ.
Stovall, W. D.....	Univ. of Wise.
Strong, Richard P.....	Harvard Univ.
Strouse, Solomon.....	Los Angeles, Calif.
Stuart, C. A.....	Brown Univ.
Stucky, C. J.....	Clinton, Conn.
Stunkard, Horace W.....	New York Univ.
Sturgis, C. C.....	Univ. of Mich.
Sugg, J. Y.....	Cornell Med. Coll.
Sugiura, Kanematsu.....	Memorial Hosp., N. Y.
Sullivan, M. X.....	Georgetown Univ.
Sullivan, Walter E.....	Univ. of Wise.



Sulzberger, Marion B.....	N. Y. Post-Graduate Med.
Sumner, F. B.....	Scripps Inst. of Oceanography
Sumner, James B.....	Cornell Univ.
Sundstroem, Edward S.....	Univ. of Calif.
Sure, Barnett .....	Univ. of Arkansas
Swann, Howard G.....	Univ. of Chicago
Swanson, W. W.....	Univ. of Chicago
Swartzwelder, J. C.....	Louisiana State Univ.
Sweany, Henry C.....	Chicago, Ill.
Sweeney, Henry M.....	Univ. of S. Dakota
Sweet, J. Edwin.....	Cornell Univ. Med. Coll.
Swett, Francis H.....	Duke Univ.
Swift, H. F.....	Rockefeller Inst.
Swindle, P. F.....	Marquette Univ. Med.
Swingle, W. W.....	Princeton Univ.
Sydenstricker, V. P.....	Univ. of Georgia Med.
Syverton, Jerome T.....	Univ. of Rochester
<b>T</b> ainter, Maurice L.....	Stanford Univ.
Tanner, Fred W.....	Univ. of Ill.
Tashiro, Shiro.....	Univ. of Cincinnati
Tatum, A. L.....	Univ. of Wis.
Tauber, Henry.....	New York City
Tauber, O. E.....	Iowa State Coll.
Templeton, Roy D.....	Loyola Univ.
Ten-Broeck, Carl.....	Rockefeller Inst., Princeton, N. J.
Thalhimer, William .....	N. Y. City
Thayer, S. A.....	St. Louis Univ.
Thienes, Clinton H.....	Univ. of S. Calif.
Thomas, Arthur W.....	Columbia Univ.
Thomas, J. E.....	Jefferson Med. Coll.
Thomas, Karl.....	Physiologisch-Chemisches Inst., Leipzig
Thomas, Walter S.....	Rochester, N. Y.
Thompson, Kenneth W.....	Yale Univ.
Thompson, Richard.....	Coll. of Phys. and Surg., N. Y.
Thompson, Willard O.....	Rush, Med. Coll.
Thorp, Frank, Jr.....	Michigan State Coll.
Tillett, W. S.....	N. Y. Univ. Med.
Tisdall, Frederick F.....	Univ. of Toronto
Tocantins, L. M.....	Jefferson Med. Coll.
Tolstoi, Edward .....	N. Y. City
Tomesik, Joseph .....	Budapest, Hungary
Toomey, J. A.....	Western Reserve Univ.
Torrance, C. C.....	Jamestown, N. Y.
Torrey, Harry B.....	Children's Hosp., Oakland, Calif.
Torrey, J. C.....	Cornell Univ. Med. Coll.
Trattner, H. R.....	Western Reserve Univ.
Travell, Janet.....	Cornell Univ. Med. Coll.
Travis, L. E.....	Univ. of S. Calif.
Tripoli, C. J.....	Louisiana State Univ.

Tsen, Edgar T. H.	Epidemic Prevention Bureau, China
Turner, C. D.	Northwestern Univ.
Turner, Charles W.	Univ. of Mo.
Turner, Edward L.	Meharry Med. Coll., Nashville, Tenn.
Turner, Kenneth B.	Presbyterian Hosp., N. Y. City
Turner, Robert G.	Brandon, Wis.
Turner, Roy H.	Tulane Univ.
Tuttle, W. W.	State Univ. of Iowa
Tweedy, W. R.	Loyola Univ. Med.
Tyler, D. B.	Calif. Inst. of Technology

Uber, Fred M.	Univ. of Mo.
Uhlenhuth, Edward	Univ. of Maryland
Uyei, N.	Hormone Research Inst., Japan

Van Allen, C. M.	New Haven, Conn.
Van Dyke, H. B.	E. R. Squibb and Sons
Van Harreveld, A.	Calif. Inst. of Technology
Van Liere, E. J.	Univ. of W. Va.
Van Slyke, Donald D.	Rockefeller Inst.
Van Wagenen, Gertrude	Yale Univ.
Van Winkle, W., Jr.	Food and Drug Administration
Vars, Harry M.	Univ. of Pa.
Vaughan, Stuart L.	Buffalo Gen. Hosp.
Verder, Elizabeth	Nat. Inst. of Health, Washington
Vickery, H. B.	Conn. Agr. Exp. Sta.
Victor, Joseph	Research Div. of Chronic Diseases, N. Y.
Virtue, R. W.	Univ. of Denver
Visscher, J. Paul	Western Reserve Univ.
Visscher, Maurice B.	Univ. of Minn.
Voegtlin, Carl	Nat. Inst. of Health, Bethesda, Md.
Vogel, Karl M.	St. Luke's Hosp., N. Y.
Von Brand, Theodor	Catholic Univ., Washington, D. C.
Von Haam, Hans E.	Ohio State Univ.
Von Oettingen, W. F.	Bethesda, Md.

Wade, Nelson J.	St. Louis Univ.
Wadsworth, Augustus B.	N. Y. State Dept. of Health
Wakerlin, G. E.	Univ. of Ill. Med.
Waksman, S. A.	N. J. State Agr. Exp. Sta.
Walker, A. W.	Northwestern Univ.
Walker, E. L.	Univ. of Calif.
Wallace, George B.	N. Y. Univ. Med. Coll.
Wallin, Ivan E.	Univ. of Colo.
Walters, Orville S.	Central Coll., McPherson, Kan.
Walton, D. C.	Annapolis, Md.
Walton, Robert P.	Univ. of Mississippi
Walzer, Matthew	Brooklyn Jewish Hosp.
Wang, Chi Che.	Chicago, Ill.
Wang, S. C.	Northwestern Univ.
Wangensteen, Owen H.	Univ. of Minn.

Warner, E. D.	State Univ. of Iowa
Warren, Shields	Harvard Med.
Wasteneys, H.	Univ. of Toronto
Watson, Cecil J.	Univ. of Minn.
Wearn, J. T.	Lakeside Hosp., Cleveland, Ohio
Weber, Clarence J.	Univ. of Kansas Med.
Webster, Bruce	Cornell Univ. Med. Coll.
Webster, J. P.	Presbyterian Hosp., N. Y.
Webster, Leslie T.	Rockefeller Inst.
Wedd, Alfred M.	Univ. of Rochester
Wedum, Arnold G.	Univ. of Cincinnati
Weech, A. A.	Coll. of Phys. and Surg., N. Y.
Weil, Alfred J.	Pearl River, N. Y.
Weil, Arthur	Northwestern Univ.
Weinberg, Samuel J.	Univ. of Calif., L. A.
Weinman, D.	Harvard Med.
Weiskotten, Herman G.	Syracuse Univ.
Weiss, Charles	Mt. Zion Hosp., San Francisco
Weiss, Emil	Univ. of Ill. Med.
Weiss, Harry	Columbia Univ.
Weiss, Paul A.	Univ. of Chicago
Weiss, Soma	Peter Bent Brigham Hosp.
Welch, A. D.	Glenolden, Pa.
Weld, Julia T.	Columbia Univ.
Welker, W. H.	Univ. of Ill. Med. Coll.
Weller, Carl V.	Univ. of Mich.
Wells, L. J.	Univ. of Minnesota
Wenner, William F.	Kalamazoo, Mich.
Werkman, Chester H.	Iowa State Coll.
Werner, A. A.	St. Louis Univ.
Werner, S. C.	Coll. of Phys. and Surg.
West, Edward S.	Univ. of Oregon Med.
West, Randolph	Presbyterian Hosp., N. Y.
Wetzel, Norman C.	Western Reserve Univ.
Weymouth, Frank W.	Stanford Univ.
Wheeler, Mary W.	N. Y. State Dept. of Health
Whipple, George H.	Univ. of Rochester
Whitaker, D. M.	Stanford Univ.
White, Abraham	Yale Univ.
White, H. L.	Camp Robinson, Ark.
Whitehead, Richard W.	Univ. of Colo.
Wible, Charles L.	Univ. of Nebraska
Wiebe, A. H.	Norris, Tenn.
Wiener, A. S.	Jewish Hosp., Brooklyn
Wiersma, C. A. G.	Calif. Inst. of Technology
Wiggers, Carl J.	Western Reserve Univ.
Wilgus, H. S., Jr.	Colo. State Coll.
Wilhelmj, C. M.	Creighton Univ. Med.
Williams, Harold H.	Children's Fund of Mich., Detroit
Williams, Horatio B.	Columbia Univ.

Williams, J. R.	Highland Hosp., Rochester, N. Y.
Williams, John W.	Mass. Inst. of Technology
Williams, Robert R.	Western Electric Co.
Willier, Benjamin H.	Johns Hopkins Univ.
Willis, Henry S.	Maybury Sanitarium, Northville, Mich.
Wilson, D. Wright	Univ. of Pa.
Wilson, Frank N.	Univ. of Mich.
Wilson, J. W.	Brown Univ.
Wilson, James R.	Syracuse, N. Y.
Wilson, Robert H.	U. S. Dept. of Agri.
Windle, W. F.	Northwestern Univ.
Winslow, C.-E. A.	Yale Univ.
Winter, C. A.	State Univ. of Iowa
Winternitz, Milton C.	Yale Univ.
Wintersteiner, Oskar	Squibb Inst., New Brunswick, N. J.
Wiseman, B. K.	Ohio State Univ.
Witebsky, E.	Buffalo Gen. Hosp.
Witschi, Emil	State Univ. of Iowa
Witzemann, Edgar J.	Univ. of Wise.
Woglom, W. H.	Columbia Univ.
Wolbach, S. B.	Harvard Univ.
Wolfe, J. M.	Albany Med. Coll.
Wong, S. C.	Peiping Union Med. Coll.
Wood, Francis C.	St. Luke's Hosp., N. Y.
Woodruff, L. L.	Yale Univ.
Wright, Arthur W.	Albany Med. Coll.
Wright, Harold N.	Univ. of Minn.
Wright, Irving S.	Cornell Univ. Med. Coll.
Wu, Hsien	Peiping Union Med. Coll.
Wulzen, Rosalind	Oregon State Coll.
Wyckoff, R. W. G.	Lederle Lab., Pearl River, N. Y.
<b>Y</b> atsu, Naohide	Zoological Inst., Tokyo, Japan
Yeager, J. F.	Beltsville, Md.
Yerkes, Robert M.	Yale Univ.
Yonkman, F. F.	Wayne Univ. Med.
Youland, William E., Jr.	N. Y. Med. Coll.
Youmans, W. B.	Univ. of Oregon Med.
Young, C. C.	Dept. of Health, Mich.
Young, William C.	Orange Park, Fla.
Youngburg, Guy E.	Univ. of Buffalo Med.
Yudkin, Arthur M.	Yale Univ.
<b>Z</b> eckwer, Isolde T.	Univ. of Pa.
Zerfas, L. G.	Camby, Ind.
Zia, S. H.	Peiping Union Med. Coll.
Ziegler, M. R.	Univ. of Minn.
Zimmerman, Harry M.	Yale Univ.
ZoBell, Claude E.	Scripps Inst. of Oceanography
Zondek, Bernhard	Jerusalem, Palestine
Zucker, Theodore F.	Columbia Univ.
Zwemer, R. L.	Coll. of Phys. and Surg., N. Y.

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